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**PROCEEDINGS OF THE FOURTEENTH CONFERENCE ON ENVIRONMENTAL
TOXICOLOGY 15, 16, and 17 NOVEMBER 1983**

**UNIVERSITY OF CALIFORNIA, IRVINE
OVERLOOK BRANCH, P.O. BOX 31009
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

A handwritten signature in black ink, appearing to read "Bruce Stuart", with a stylized flourish at the end.

BRUCE O. STUART, PhD
Director Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

ERRATA SHEET

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PREFACE

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WELCOMING REMARKS

Billy E. Welch, Ph.D.
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Good morning, ladies and gentlemen. It is a pleasure for me to welcome you on behalf of the Air Force Aerospace Medical Division to this 14th Conference on Environmental Toxicology. The sponsor for this year's meeting is the University of California at Irvine, under the terms of the contract with the Air Force Aerospace Medical Research Laboratory and with the support of the Toxicology Detachment of the Naval Medical Research Institute.

Over the years that these conferences have been in existence, there has been one unifying theme and purpose: "The assembling of knowledgeable people to discuss current toxicologic research issues in a forum designed to promote a free and honest exchange of ideas." The past conferences have indeed been successful in achieving the desired goals and, based on my review of the topics and attendees of this conference, I believe the 14th Conference will assume its rightful place with its predecessors.

It is interesting as the years have passed to note the changes that have occurred in the content of these conferences as well as in the general field of toxicology. The Air Force and the Navy have maintained an active interest in toxicology for many years. We like to believe that this interest has been a dynamic one and that our program has evolved in terms of both scientific content and relevance to our missions. For example, in 1972, the major emphasis of the conference was on the toxicology of halogenated compounds (found in solvents, aerosol propellants, and fire extinguishants) and on the toxicology of pyrolysis and rocket exhaust products. Discussion of mechanisms was relatively sparse, with most of the attention being devoted to documentation of effects and development of instrumentation. In 1977, in contrast, the focus of the conference was much broader and involved a number of papers defining basic mechanisms in toxicology as well as highlighting the true, multidisciplinary aspects of modern toxicology. The topics that year covered not only the mechanisms aspects of toxicology, but also dealt with experimental, occupational, and environmental toxicology. Each of the conferences, as in the two years cited, has attempted to focus on problems not only of Air Force and Navy interest, but also of national interest. Each conference has continued to build on the past and, I believe, has contributed to and is representative of the spectacular growth and sophistication present in the field of toxicology.

This year's conference is no exception. Our understanding of the nervous system is expanding, and the interest that exists in neurotoxicity is certainly reflective of not only the trend in

toxicology per se, but in the direction and interest of our mission. The ever-increasing complexity of weapon systems and the demands placed on those that operate and maintain these systems dictate our interest and concern about neurotoxicity and the possible impact that this toxicity might have on performance. Environmental concerns continue to be of great importance. The real world is a complicated toxicologic milieu, requiring that we be capable of addressing the toxicologic impact of mixtures of various compounds with all of the attendant problems of dealing with mixtures. Obviously, one of the keys that will make it possible to deal with the mixture problem will be our ability to model and simulate, thus the interest in pharmacokinetics and the development of the basic data to permit this to be accomplished. Finally, we must be concerned about the translation and application of these data to the work place. Computerization of data from all sources (work place, environment, and laboratory) has made it possible to provide almost "real-time" toxicologic support. Future demands upon toxicology will be even greater than in the past and, in my opinion, will require the development of systems that will be predictive in nature rather than merely documenting past events.

Today, as perhaps at no time in the past, we are confronted with more and more complicated toxicologic puzzles. The field has moved light years beyond the early days of survival or gross pathology as the end point. Compounds that were once thought to be safe are now being found to be hazardous. Hazardous compounds that were once properly disposed of are now appearing in drinking water supplies. Standards that were once known to be adequate are now being questioned. Ralph Wands, in his closing remarks at the 8th Conference on Environmental Toxicology said: "Toxicology, as any science, can only deal with defining the risks; it cannot make the ultimate social decision of how much risk is acceptable." That statement is still true today. What has changed, however, is that scientists are now actively involved in both defining risk and determining its acceptability. This places a heavy burden on each of us to assure that our scientific objectivity is not adversely influenced by our involvement in the social questions. Your presence here today attests to your interest in maintaining this objectivity.

Again, I welcome you to this 14th Conference. Thank you.

INTRODUCTORY ADDRESS

Anthony A. Thomas, M.D.
SES (Retired)

Ladies and Gentlemen,

It's good to see so many old friends and familiar faces again at this 14th Conference on Environmental Toxicology.

As many of you know, I have had a long association with these annual meetings; as a matter of fact, I am to blame for instigating the need for these, way back in 1961. There was a clear need for timely and spontaneous exchange of research findings among government, university, and industrial toxicology laboratories so that we could keep abreast of the rapid developments in aerospace and environmental toxicology.

At the beginning, there was a truly large scale chemical synthesis effort underway for finding new, high-energy storable liquid and solid rocket and missile propellants, - kind of a "chemical roulette". This dictated the need for protecting our research chemists who were potentially exposed to, albeit small quantities but highly reactive, intermediates and end products at the bench (or, hopefully, around the hood).

By the time this wheel of fortune slowed down (because what looked good on a theoretical thermodynamics basis may not have stood the acid tests of storability, material compatibility, specific impulse during static test firings of small motors, etc.), the "Man-In Space" program was ready for a quantum leap, and the country reached out for the moon.

Those were heady and exciting times, fraught with monumental uncertainties about man's tolerance to artificial atmospheres of pure oxygen or mixed oxygen/nitrogen environments at reduced barometric pressures. Added to these were the enhanced evolution of volatile contaminants from cabin materials and man himself in a hermetically sealed atmosphere.

Because of weight considerations, air purification equipment had its limitations in size and capacity, calling for new approaches to the problems of environmental toxicity during long term, truly continuous around-the-clock exposure of astronauts.

As the boosters got larger and more powerful, there came the chore of preventing serious contamination of the environment of this spaceship earth on which you and I are destined to live, together with our limited natural resources, our flora and fauna, our air, water, and soil.

Venting of propellant vapors from large storage tanks, the dangers of accidental spills and transportation mishaps and the routine disposal of waste materials had to be controlled or decontaminated to limit effects on health and environment, at least to a degree, to where they are still reversible.

Many times the question was asked, why is the Air Force in the toxicology business? Why not let the health agencies conduct the necessary research for the Department of Defense? The answer was, and still is, simple: priorities in accomplishing the work. It must be done expeditiously, with a quick reaction capability to clean up any mess we may have created unknowingly, if and when it happened.

As methodologies became more sophisticated so did our process in the extrapolating of toxicity data to human health effects. The key here is kinetic information on what happens to these compounds in the body and how the human organism metabolizes, detoxifies, and eliminates the parent compound and its breakdown fragments.

This process of extrapolation requires the team work of many scientific disciplines, starting with analytical and biological chemistry, leading through pharmacology, toxicology, pathology, occupational medicine, statistics, epidemiology, industrial hygiene, environmental sciences, etc., just to name a few.

So, if you look at the program for these 2 1/2 days, you see this team effort at work.

The first day has as its theme peripheral neurotoxicity of hydrocarbons and organophosphorus compounds, a topic of great interest to university researchers, industrial hygienists, and physicians, and of course to Department of Defense operations.

On the second day there are two important topics. First, toxicity to the male reproductive system and the knotty problem of toxicity from exposure to complex mixtures.

The third morning we will attempt to hammer out a reasonable approach to how to put all this manifold toxicological information to use in the field, within the framework of an occupational health management system to protect our most valuable resource, the worker in the chemical environment, and of course all of us live in a complex chemical environment even when not at work.

You will also note that each session has also either a tutorial or a fundamental review type of lecture, to help us who happen to be on a slightly different wavelength from the man at the rostrum. And even more important in a true adherence to the legacy of these annual conferences, we ask you to be an active participant in our open forums, where the real exchange of ideas will originate.

You will have to forgive me if I have sounded like an old man, reminiscing about the good old times. My only excuse is that I am still not too old to get excited about the challenges we have conquered, and the new ones awaiting us.

It was my privilege to play a role in this exciting age of development, and my good fortune to have had a large scientific pool of talent to draw on, both inhouse at the Aerospace Medical Research Laboratories and at the many campuses of the University of California, to meet these challenges.

Of course, I have been in retirement for two years but as the German proverb has it "Alte Liebe rostet Nicht..." (Old Loves Never Die). Neither will I stop loving this exciting discipline of toxicology.

SESSION I

HEXANE NEUROTOXICITY

Chairman

**Peter S. Spencer, Ph.D.
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NEUROTOXICOLOGY: A NEW SCIENTIFIC CHALLENGE

Peter S. Spencer, Ph.D., M.R.C. Path.

Professor of Neuroscience
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INTRODUCTION

Neurotoxicology is the study of actions of agents that cause adverse effects on the nervous system and behavior of humans and animals. This definition encompasses toxic chemicals of diverse origins, includes adverse effects traceable to changes in structure or function of the nervous system, special sense organs and neuroendocrine system, and recognizes the importance of conjoint study of mechanisms and effects of neurotoxins in humans and animals, experimental or otherwise. Because of the chemical complexity, delicate internal balance and enormous biological responsibility of the nervous system, chemicals with potentially neurotoxic properties are legion. Neurotoxicity is, perhaps, one of the most common types of toxic response recognized in Man.

Factors Regulating Neurotoxic Response

The net effect produced by an individual chemical present in blood depends on many factors. Ability to enter the nervous system, specific site of attack within neural tissue, concentration of the proximate toxin at the target site, chemical reactivity of this agent, and duration of exposure to target site are some of the major considerations. All of these factors are heavily influenced by circumstances surrounding exposure, the portal of body entry, the distribution within tissues, the rate of breakdown, the toxicity of metabolic products, and the efficacy of excretion. The present state of knowledge of these critical factors is limited for all but a few neurotoxic substances, and it is often difficult to predict whether a particular chemical is likely to be neurotoxic or not. The more we know about these factors in relation to any one chemical, the more accurate the judgment of neurotoxic response and the prediction of risk.

Dose and duration of exposure are pivotal concepts in determining whether an agent will produce a neurotoxic effect. Chemical agents which at certain doses elicit a profound, long-lasting response, may be harmless at low levels, or may even be required for normal nervous system function. This is well illustrated by recent experience with human exposure to vitamin B₆, whereas

small, steady quantities are mandatory for normal functioning of the nervous system; excessive amounts cause axonal degeneration and sensory neuropathy.

Duration of exposure to a particular concentration of a chemical substance plays a major role in determining whether that substance is neurotoxic and whether the pathophysiologic change is reversible. The effect of alcohol consumption is a familiar example, whereas a single large dose induces a state of drunkenness -- a totally reversible neurotoxic state; repeated doses over prolonged periods elicit not only the acute response but also chronic degenerative brain disease.

When evaluating neurotoxic potency, differential individual susceptibility to chemical agents with potentially adverse effects must be considered. The principle of individual susceptibility is well known in clinical neurology where toxic side effects of certain therapeutic agents appear only in a sub-group of patients. Such individuals will commonly show an inherited trait that affects the rate of disappearance of the drug. Other population sub-groups that may be specially vulnerable to neurotoxic agents (although present evidence is limited) are the elderly and the young. Strong evidence of susceptibility to neurotoxic agents exists for individuals with compromised liver or kidney function. Others with special sensitivity to neurotoxic agents include those who have metabolic diseases, such as diabetes mellitus, that by themselves compromise nervous system function. Taken together, these considerations bear on the determination of acceptable levels of human exposure to chemical agents.

Types of Neurotoxic Disease

The effects of neurotoxic agents can be classified according to their apparent sites of action. This provides a rational approach to understanding the clinical consequences of exposure and a starting point to an enquiry directed at mechanism(s) of toxicity. The basic cellular components of the nervous system--neuron and axon, glial cells, and myelin--form the basis for this classification system. Those chemicals affecting neurons may (a) perturb neuroelectrical transmission by altering the function of ionic channels in excitable membranes, (b) disrupt the integrity of neurotransmitter or neurohumeral systems, or (c) cause structural breakdown of axon or neuronal perikaryon. Other targets of neurotoxic chemicals include myelin or myelin-producing cells, astrocytes, muscle, and neural vasculature. Note that an agent may have more than one site of action and that this is commonly related to dose or rate of intoxication. For example, large doses of acrylamide appear to produce encephalopathy, ataxia, depletion of Purkinje cells, and subsequently, axonal neuropathy; smaller doses spread over a longer period of time also lead to axonal neuropathy while sparing Purkinje cells.

Occurrence of Neurotoxic Disease

Most human neurotoxic disease in the United States is iatrogenic in origin and results from prolonged treatment with anti-convulsants, anticholinergics, neuroleptics, antiparkinsonian and antineoplastic drugs. However, neurotoxic disease is encountered in many other settings. Substance abuse, particularly involving ethanol, narcotics, hallucinogens, central stimulants, solvents, and nitrous oxide, leads to short or more longer lasting neurological dysfunction. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), an impurity in a synthetic psychoactive drug that produces rapid-onset parkinsonism, is a recent example of the latter phenomenon. In the occupational setting, the acute cholinergic crisis associated with pesticide overexposure is commonplace among pesticide workers, and there is concern that these agents may produce long-lasting, subtle alterations of behavior and memory. Many other substances found in the workplace have been indicted in neurological illnesses ranging from polyneuropathy to organic brain syndrome. Environmental pollutants with neurotoxic potential are pervasive and include carbon monoxide, lead, and mercury. Neurotoxic agents in foodstuffs, present as intrinsic components, are responsible for large outbreaks of neurological disease in the Third World. For example, excessive consumption of cassava (Manihot esculenta) or the chickling pea (Lathyrus sativus) precipitates spastic paraparesis. Adulterants present in food also have been held responsible for neuromuscular disease, the Spanish "Toxic Oil Syndrome," a phasic multisystem disease which led to global neuromuscular atrophy in the more severely affected, being the most recent example. Mycotoxins still contaminate food for human consumption in certain parts of the world, ergot and aflatoxin are two of several associated with neurological disease. Other important biological neurotoxins are those associated with botulism, tetanus, and diphtheria. Numerous animals secrete or harbor potent toxins, including agents that disturb nerve conduction and synaptic transmission. Neurotoxic substances such as arsenic and thallium compounds occasionally are deliberately added to food for homicidal or suicidal purposes. Chemicals with experimentally proven neurotoxic potential also exist as food additives (monosodium glutamate), flavors, and fragrances (2,6-dinitro-3-methoxy-4-t-butyltoluene) and certain anti-seborrheic agents (zinc pyrithione). Substances with potential neurotoxic properties are therefore encountered in food, air, and water, in the general environment, and in domestic and occupational settings.

Conclusion

The science of neurotoxicology is in its infancy. The scale of the problem has been recognized, the scope of the discipline defined, and a preliminary classification of neurotoxic response developed. Some information is available on the relative frequency of neurotoxic disease in developed and developing countries, and in which environmental or social niches these diseases

occur. The challenge facing basic and clinical neurotoxicologists is to understand how these diseases develop and how they can be prevented. Since many neurotoxic conditions mimic naturally occurring neurological diseases, investigations designed to determine the biological actions of neurotoxic chemicals will undoubtedly illuminate other types of nervous-system compromise. Viewed from this perspective, the neurotoxic agent is not only a threat to human health, but also a powerful investigatory tool.

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REVIEW OF THE TOXICOKINETICS OF n-HEXANE

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INTRODUCTION

n-Hexane is used extensively both in industry and in commerce. It is usually sold as a mixture of C6 isomers and is rarely used in large volumes as a pure compound. In an occupational setting, few untoward effects have been reported for n-hexane when adequate industrial hygiene practices have been followed. In some industries, however, industrial hygiene controls were often less than adequate and as a result several cases of peripheral neuropathy have occurred due to exposure to n-hexane. The neurotoxic effects of n-hexane were not fully realized until an outbreak of neuropathy occurred in workers exposed to methyl n-butyl ketone, a solvent similar in structure to that of n-hexane (Billmaier et al., 1974). Subsequent investigations revealed that the metabolism of n-hexane was similar to that of methyl n-butyl ketone and that both compounds were converted to common neurotoxic metabolites (DiVincenzo et al., 1976, 1977). The objective of this presentation is to review the toxicokinetics of n-hexane as it relates to occupational and consumer exposures. A thorough review of this subject matter has been reported elsewhere (Spencer et al., 1980).

ABSORPTION

n-Hexane is a volatile lipophilic solvent that may be absorbed through pulmonary, oral, and dermal routes.

PULMONARY ABSORPTION

Several investigators have demonstrated that exposure to high concentrations of n-hexane vapor has produced toxicity in experimental animals, indicating that n-hexane is readily absorbed through the lungs (Sandmeyer, 1981). In 1974, Nomiyama and Nomiyama studied the pulmonary absorption, retention, and excretion of n-hexane by volunteers. Experimental exposures were carried out for 4 hours at vapor concentrations ranging from 87 to 122 ppm. The pulmonary uptake of n-hexane was estimated to be about 28% of the inhaled concentration. About 80% of the

absorbed n-hexane was eliminated unchanged in expired air and 20% was retained by the body. Similar results were reported by Brugnone et al. (1978).

These studies indicate that the pulmonary uptake of n-hexane may be limited by its solubility in blood. Up to 30% of the inhaled vapor is likely to be absorbed through the lungs and that which is absorbed is largely eliminated unchanged in the expired air.

GASTROINTESTINAL ABSORPTION

Common symptoms encountered when n-hexane has been accidentally ingested include nausea, vertigo, bronchial and intestinal irritation, and a variety of CNS effects. It is estimated that the ingestion of about 50 grams of n-hexane would be fatal to humans (Sandmeyer, 1981). There are numerous studies in which n-hexane has been administered by gavage to experimental animals to induce toxic effects. Collectively, these studies demonstrate that n-hexane is readily absorbed from the gastrointestinal tract.

PERCUTANEOUS ABSORPTION

The dermal application of 2-5 mL of n-hexane per kg of body weight to rabbits for 4 hours produced symptoms characteristic of n-hexane toxicity (Sandmeyer, 1981). n-Hexane is lipophilic and is expected to be absorbed through the skin.

TISSUE DISTRIBUTION

Several investigators have demonstrated that n-hexane is widely distributed throughout the tissues of the body. Böhlen et al. (1972) studied the tissue distribution of n-hexane in rats exposed for 2-10 hours to about 50,000 ppm of hexane vapor. Tissue saturation of hexane occurred within 4-5 hours of exposure in all tissues examined with the exception of liver. The concentration of n-hexane in the liver increased linearly with time and did not reach saturation at 10 hours. The authors concluded that there was a direct proportionality between the tissue saturation of n-hexane and the lipid content of the tissues examined.

Bus et al. (1981) exposed male Fischer 344 rats to 1000 ppm n-hexane for 6 hours. At various times after the exposure, rats were killed and tissues were removed and analyzed for n-hexane. n-Hexane was detected in all tissues examined. The highest concentration of n-hexane was found in nerve tissue. In addition, n-hexane was converted to metabolites that were detected in each of the above tissues.

In another study, Bus et al. (1982) exposed male Fischer 344 rats to 500, 1000, 3000, or 10,000 ppm [^{14}C] n-hexane for 6 hours and determined the distribution of radioactivity in tissues 72 hours after the exposure. The concentration of radioactivity was

highest in the liver and the kidneys and did not increase linearly with the exposure concentration. This non-linearity was most apparent at 10,000 ppm.

Bus et al. (1979) exposed pregnant rats to 1000 ppm of n-hexane for 6 hours per day during gestation. The concentration of n-hexane and its metabolites in the fetus were comparable to those concentrations found in maternal blood after the exposure. In this study the authors concluded that n-hexane was not teratogenic and had a minimal potential to affect prenatal development.

METABOLISM

During the late nineteen sixties and mid-nineteen seventies, several investigators demonstrated that n-hexane induced the hepatic mixed function oxidase system in experimental animals. The specific activities of cytochrome P₄₅₀, cytochrome b₅, cytochrome P₄₅₀ reductase, and UDP glucuronyltransferase were increased following treatment with n-hexane (McLean, 1967; Kramer et al., 1974; Vainio, 1974; Notten and Henderson, 1975a, 1975b). In microsomal studies, it was shown that n-hexane was hydroxylated in 3 positions leading to the formation of 1-, 2-, or 3-hexanol (Kramer et al., 1974). The major metabolite was 2-hexanol.

During the mid-1970s occupational exposure to methyl n-butyl ketone led to an outbreak of peripheral neuropathy in a coated fabrics plant located in Columbus, Ohio (Billmaier et al., 1974). We investigated the metabolism of methyl n-butyl ketone in guinea pigs and proposed the metabolic pathway illustrated in Figure 1.

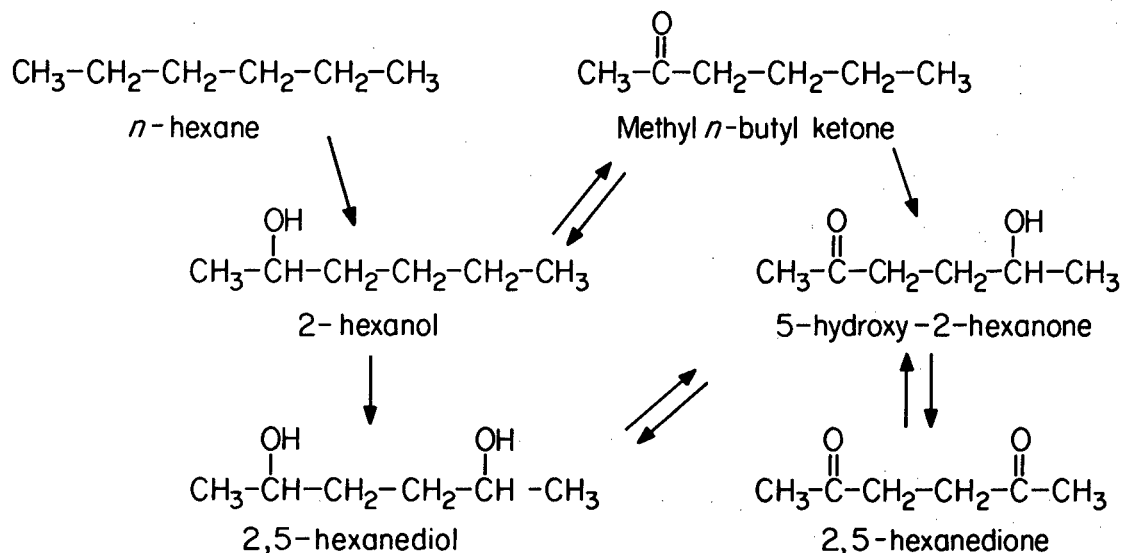


Figure 1. Partial metabolic pathway for n-hexane and methyl n-butyl ketone (taken from DiVincenzo et al., 1976, Toxicol. Appl. Pharmacol., 36:54).

Methyl n-butyl ketone was metabolized to either 5-hydroxy-2-hexanone or to 2-hexanol which in turn could be converted to 2,5-hexanedione or 2,5-hexanediol (DiVincenzo et al., 1976). Since n-hexane was also reported to be neurotoxic in man (Yamada, 1964), the metabolism of n-hexane was also investigated. n-Hexane was converted to the same metabolites that were identified for methyl n-butyl ketone. Both n-hexane and methyl n-butyl ketone were converted to 2,5-hexanedione, the metabolite responsible for the neurotoxicity of these solvents. Similar findings were reported by Couri et al. (1978).

We subsequently investigated the metabolism of methyl n-butyl ketone in greater detail and related these findings to the metabolism of n-hexane. A composite metabolic pathway for n-hexane is illustrated in Figure 2. n-Hexane is oxidized by the

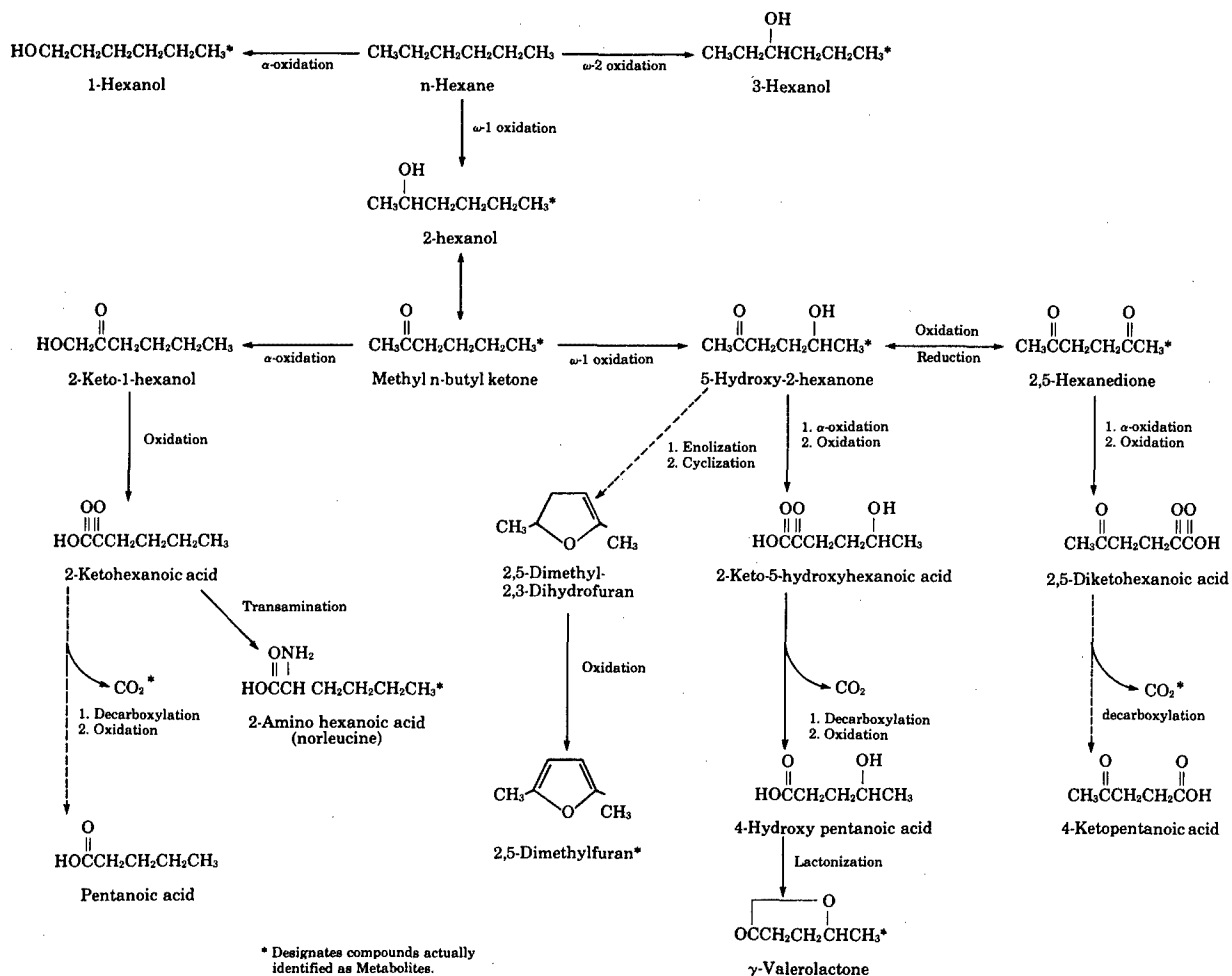


Figure 2. Proposed metabolic pathway for n-hexane (adapted from the works of Kramer et al., 1974, Chemo-Biol. Interact. 8:11; DiVincenzo et al., 1977, Toxicol. Appl. Pharmacol., 41, Couri et al., 1978, Am. Ind. Hyg. Assoc. J., 39:295; and Angelo and Bischoff, 1982, Air Force Aerosp. Med. Res. Lab., AFAMRL-TR-81-149, 250, AD A115 900).

hepatic mixed function oxidase system to 1-, 2-, or 3-hexanol. 2-Hexanol is converted to methyl n-butyl ketone which then can undergo α -oxidation to produce norleucine and pentanoic acid. Pentanoic acid enters intermediary metabolism. In addition, methyl n-butyl ketone is converted to 2,5-hexanedione. 5-Hydroxy-2-hexanone, an intermediate in this pathway, can give rise to 2,5-dimethylfuran and λ -valerolactone (DiVincenzo et al., 1976 and 1977).

These same metabolites have been identified in the urine of workers exposed to n-hexane vapor, verifying that the same metabolic pathways that were found in the rat were also found in man (Perbellini et al., 1981; Iwata et al., 1983).

Most recently the dose dependent disposition of [^{14}C]n-hexane was investigated in rats exposed to 500, 1000, 3000, or 10,000 ppm of n-hexane vapor (Bus et al., 1982). n-Hexane was eliminated in expired air unchanged and as $^{14}\text{CO}_2$. The remainder of the radioactivity was eliminated in urine presumably in the form of metabolites. The percentages of total radioactivity recovered in the tissues and carcasses were lower at the higher exposure concentrations than were found at the lower exposure concentrations.

The relative neurotoxicity of n-hexane and its metabolites was investigated by Krasavage et al. (1979). 2,5-Hexanedione was the most potent neurotoxin tested and required about 17 days to produce clinical signs of neuropathy. The other hexacarbon metabolites, 5-hydroxy-2-hexanone, 2,5-hexanediol, methyl n-butyl ketone and 2-hexanol, were also neurotoxic, presumably due to their conversion to 2,5-hexanedione. n-Hexane did not produce a neuropathy in this study. The onset of neurotoxicity correlated directly with the serum concentrations of 2,5-hexanedione and also with the area under the serum concentration-time curve for 2,5-hexanedione. The time course for the formation and disappearance of 2,5-hexanedione in the serum of rats given equimolar doses of n-hexane and its metabolites is illustrated in Figure 3. These data support the hypothesis that the neurotoxic potency of these compounds was related to the amount of 2,5-hexanedione produced. This figure shows that very little 2,5-hexanedione was produced from n-hexane. The difference between the neurotoxic potency of MnBK and n-hexane is of interest since it has been inferred that these two compounds were of similar neurotoxic potency. These results indicate that a more extensive exposure to n-hexane would be required to produce a neuropathy that was equivalent to that seen with MnBK.

EXCRETION

The preceding studies demonstrated that n-hexane was eliminated predominantly by the pulmonary and urinary routes. Bus et al. (1982) reported that the elimination half-lives for exhaled n-hexane, following a 6-hour exposure to [^{14}C]n-hexane in rats, were biphasic. The initial phase which occurred within the first

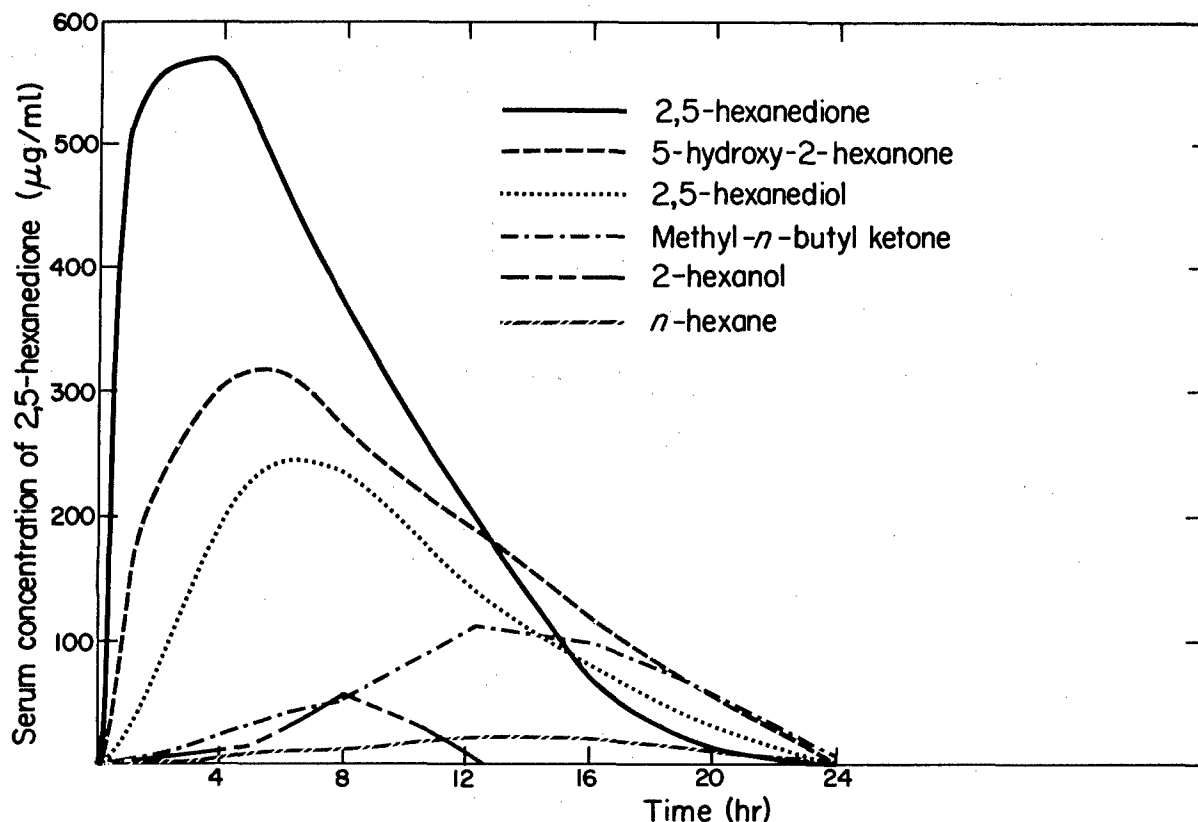


Figure 3. Time course of 2,5-hexanedione in the serum of rats dosed with 6.6 mmol/kg of each hexacarbon (taken from Krasavage et al., 1980, Toxicol. Appl. Pharmacol., 52:433).

few hours accounted for greater than 90% of the recovered n-hexane. The estimated half-lives for the α - and β -excretion phase were similar at all exposure concentrations and ranged from 0.8 to 1.4 hour for the α -phase and 4.4 to 10.9 hour for the β -phase. Elimination half-lives for radioactivity in urine and expired air were similar, and over 90% of the total radioactivity was accounted for within the first 24 hours.

SUMMARY

n-Hexane is a volatile, lipophilic solvent that is readily absorbed by the pulmonary, oral, and dermal routes. It is rapidly excreted unchanged in expired air, and is excreted in urine in the form of metabolites. n-Hexane is metabolized by a complex pathway that leads to a variety of oxidation products, one of which is 2,5-hexanedione. The neurotoxicity of n-hexane is related to its metabolism to 2,5-hexanedione. n-Hexane is the least potent member of a series of neurotoxic hexacarbons.

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INTERACTIONS OF KETONES AND HEXACARBONS

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INTRODUCTION

Methyl ethyl ketone (MEK), a widely used commercial solvent, is frequently combined with other aliphatic and aromatic hydrocarbons in paint thinners, lacquers, and solvents for technical coatings (NIOSH, 1978). Although MEK itself is considered an innocuous compound, the above products can contain n-hexane and methyl n-butyl ketone (MnBK), which are known neurotoxic solvents. While MEK itself appears devoid of neurotoxic properties (Saida et al., 1976; Spencer and Schaumburg, 1976; Altenkirch et al., 1977; Egan et al., 1980), recent clinical and experimental findings indicate that this ketone can enhance the neurotoxic properties of both n-hexane and MnBK. This property was first suspected in the 1973 outbreak of occupational neuropathy among Ohio textile workers exposed to mixtures of MEK and MnBK (Allen et al., 1974; Billmaier et al., 1974) and later demonstrated experimentally in rats continuously exposed to these mixtures (Saida et al., 1976; Couri et al., 1977). MEK-related neurotoxic potentiation was again suspected in an outbreak of n-hexane-induced neuropathy among solvent abusers (glue-sniffers,

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"huffers") in West Berlin (Altenkirch et al., 1977) and subsequently confirmed in rats exposed to high levels of both n-hexane and MEK (Altenkirch et al., 1978). The picture emerging from these studies is that MEK appears to accelerate the onset and severity of the "dying-back" neuropathy produced by hexacarbons. The mechanism underlying this potentiation is unknown.

It was our intention to replicate and investigate this interaction in tissue culture using organotypic explants of mouse spinal cord with attached dorsal root ganglia and striated muscle. At maturity, this complex displays morphological and bioelectrical features typical of mammalian neuromuscular tissues *in vivo*. This type of tissue culture has been used for many years in experimental neurobiology (Peterson and Crain, 1972) and neuropathology (Raine, 1973) and is amenable to neurotoxicological inquiries. It has been especially valuable in addressing the pathogenic and metabolic events of subchronic exposure to aliphatic hexacarbons (Veronesi et al., 1980, 1983).

MATERIALS AND METHODS

ESTABLISHMENT OF ORGANOTYPIC CULTURES

Cross sections of fetal mouse spinal cord (14 day in utero), with meningeal covering and attached dorsal root ganglia (DRG), were individually explanted onto collagen-coated coverslips. A 6-10 mm strip of skeletal muscle taken from the thigh of an adult mouse was positioned on the coverslip approximately 1 mm from the ventral cord region. Explants were provided with a drop of nutrient fluid containing Eagle's minimum essential medium with glutamine (56%), human placental serum (33%), chick embryo extract (10%), and glucose (6 mg/100 ml). The nerve-muscle complex was positioned in a sterile Maximow chamber (Maximow, 1925), sealed in its concave well, and incubated at 34-35°C. The chamber was opened twice weekly, the explant removed and washed for 10 min with Hank's basic salt solution (Gibco, Long Island, NY), fed with a fresh drop of nutrient medium, and returned to a sterile chamber. Cultures maintained in this fashion matured in approximately 8-10 weeks and were then selected for treatment by the following criteria: an agranular spinal cord with abundant myelinated central nervous system (CNS) fibers; DRG neurons housing centrally located nuclei; numerous, well-myelinated sensory and motor peripheral nervous system (PNS) fibers; and muscle tissue with well-defined cross-striations and synchronized contractions. Details of this technique are given elsewhere (Bornstein, 1973).

CHEMICALS

Test chemicals were obtained from a commercial supplier (Eastman Kodak Co., Rochester, NY) and their purity assessed by gas chromatography-mass spectrometry (courtesy of Dr. G. DiVincenzo, Eastman Kodak Co., Rochester, NY). n-Hexane was determined to be 97% pure, containing 2% methyl isobutyl ketone.

and 1% unidentified ingredients as contaminants. MEK was assessed to be 98% pure, containing 1% 2-butanol and 1% unidentified ingredients as contaminants.

TREATMENT

To establish dose:response data, n-hexane was dissolved in nutrient fluid in concentrations of 25, 50, 80, 100, or 250 ug/ml. In spite of n-hexane's low water solubility, these concentrations readily dissolved in the nutrient fluids. At least 8 mature cultures were exposed to each dose of n-hexane for periods up to 56d. Cultures were examined twice weekly for morphologic changes by brightfield microscopy. Those doses of n-hexane that induced giant axonal swellings, the morphologic hallmark of hexa-carbon neuropathology, within the exposure period were defined as "neurotoxic." The duration of exposure needed for 75% of the cultures to develop distinct axonal swellings was defined as the time-to-onset (TTO) of specific pathologic response and was recorded in days (d). Those concentrations of n-hexane which were unable to induce axonal swellings within the defined treatment period were labeled "no-response" (NR) doses.

To establish dose-response data for MEK, the solvent was dissolved in nutrient fluids in concentrations of 10, 25, 50, 200, 300, 400, or 600 ug/ml. At least 8 mature cultures were exposed to each dose of MEK for periods up to 49d. Again, individual cultures were monitored twice weekly by brightfield microscopy for morphologic changes. Concentrations of MEK that produced nonspecific cellular breakdown of the explant were defined as pancytotoxic. Noncytotoxic doses were those allowing the culture to retain normal morphology throughout the treatment period.

Neurotoxic and "no-response" doses of n-hexane were individually combined with noncytotoxic doses of MEK and dissolved in nutrient fluid in effective n-hexane:MEK mixtures of 25:50, 25:100, 50:10, 50:50, 50:100, 100:10, 100:50, 100:100, 250:25, or 250:50 ug/ml. Additional groups of mature cultures (minimum of 6 cultures per group) were individually exposed to these mixtures for periods up to 56 d. Age-matched, untreated cultures served as negative controls for all test groups. Explants were monitored microscopically and the TTO for 75% of the cultures to develop giant axonal swellings was recorded. These data were also evaluated by probit analysis (SAS, 1982) at the 28-day time point and by Cox's linear model for proportional hazards (Cox, 1972; SAS, 1982). Both models evaluated MEK, n-hexane and their multiplication product at various doses.

MICROSCOPIC EXAMINATION

Living cultures were examined within the Maximow chamber twice weekly for the appearance of distinct axonal changes. To examine the explant microscopically, a special long-working-distance, fluorite 40x oil-immersion lens with a high numerical aperture was used to penetrate optically the various glass coverslips used in the Maximow assembly. Areas of interest were photographed with Panasonic X (ASA 32) film. For electron microscopy, selected cultures were removed from their Maximow chambers, fixed in Millonig's-buffered 2.5% glutaraldehyde, followed by Millonig's-buffered 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and cleared in propylene oxide. The explant and its collagen bed were gently removed from the glass coverslip, infiltrated with epoxy-resin (Epon 812), sandwiched between two Teflon-coated coverslips (Polyscience 4505), and polymerized in a 60°C oven. This treatment produced a thin epoxy-wafer that could readily be examined with a dissecting microscope. Areas of interest were identified, trimmed from the wafer, re-embedded, and sectioned for light and electron microscopy. One-micrometer-thick epoxy sections were stained with borate-buffered 1% toluidine blue. Thin (50 nm) sections (based on interference colors) were cut with a diamond knife, collected on 200-mesh copper grids, stained with 2% uranyl acetate followed by 1% aqueous lead citrate and examined with a Zeiss 10 A transmission electron microscope.

RESULTS

CONTROL CULTURES

The nerve-muscle explants matured into structurally and functionally coupled co-cultures within 8-10 weeks. At maturation, the entire living complex measured approximately 5mm² (Figure 1). Typically, a mature control culture was composed of an agranular spinal cord containing neurons and numerous thinly myelinated CNS fibers. Clearly defined dorsal and ventral spinal roots combined to form sensory-motor nerve tracts. Large DRG (sensory) neurons contained prominent, centrally located nuclei and a cytoplasm rich in Nissl substance and other organelles. Heavily myelinated PNS fibers exhibited well-defined nodes of Ranvier and Schmidt-Lanterman clefts. Numerous unmyelinated fibers were grouped with myelinated fibers in small fascicles delimited by cells of fibroblast-type (Figure 2). Skeletal muscle tissue, which initially had been incorporated into the system as adult tissue, degenerated, regenerated, and eventually differentiated into finely striated, synchronously twitching muscle fibers. The morphologic and physiologic viability of the muscle tissues in vitro depended on motor fiber innervation. By electron microscopy, PNS and CNS myelinated axons typically contained 10-nm neurofilaments, 24-nm microtubules, longitudinally oriented mitochondria, smooth endoplasmic reticulum, and scattered vesicles. Small groups of unmyelinated axons were



Figure 1. Mature organotypic co-culture of spinal cord (c) and muscle (m) explants. Note fascicles of peripheral nerve fibers (arrow). Brightfield, living culture X20.

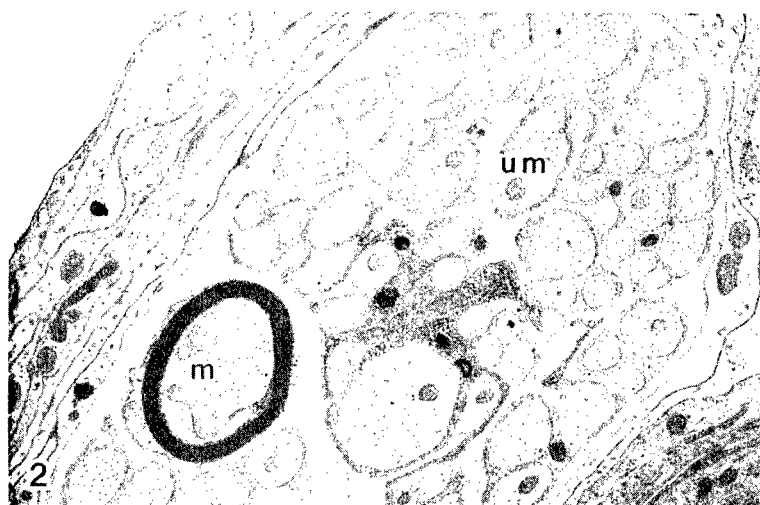


Figure 2. Peripheral nerve fascicle from control culture showing myelinated (m) and unmyelinated (um) axons. Electron micrograph X9600.

partially or completely enclosed in Schwann cell cytoplasm and typically contained a higher proportion of microtubules to neurofilaments relative to their myelinated counterparts.

n-HEXANE-TREATED CULTURES

Cultures treated with neurotoxic doses of n-hexane developed giant axonal swellings that first appeared in distal paranodal regions of large-diameter nerve fibers (Figure 3). A progression of axonal pathology was recorded in these fibers that included nodal elongation, swelling of the paranodal axon, retraction of paranodal myelin, and eventual degeneration of the fiber length distal to the swelling. By electron microscopy, the giant axonal swelling was characterized by an accumulation of 10-nm neurofilaments, peripheral displacement of neurotubules and mitochondria, and a disproportionately thin or absent myelin sheath. The pathogenesis of hexacarbon neuropathy in vitro has been detailed elsewhere (Veronesi et al., 1980, 1983).

Giant axonal swellings of this type were seen in cultures exposed to n-hexane in concentrations of, or greater than, 100 ug/ml. The duration of time (TTO) required for axonal swellings to develop in 75% of explants treated with 100 and 250 ug/ml (neurotoxic levels) was 43 and 28 d, respectively. Axonal swellings were not seen in cultures treated with 25, 50, or 80 ug/ml (NR levels) for periods up to 56 d. These data are summarized in Table 1.

TABLE 1. DOSE RESPONSE FOR n-HEXANE^a

<u>n-Hexane</u> <u>(ug/ml)</u>	<u>Response</u> <u>(TTO)</u>	<u>Incidence</u> <u>of Effects^b</u>
25	NC	0/8
50	NC	0/8
80	NC	0/8
100	43	6/8
250	28	8/8

^a Explants (8 per dose) were treated for 56 days with 25-250 ug/ml of n-hexane dissolved in nutrient fluid.

^b Tissue treated with 100 ug/ml displayed no pathological change (NC). Explants treated with doses of 100 ug/ml or greater developed giant axonal swellings in nerve fibers at the times stated in days. TTO: Time-to-onset of axonal swellings in 75% of cultures.

MEK-TREATED CULTURES

Organotypic explants treated with 600 ug/ml of MEK displayed a generalized cellular breakdown (pancytotoxic response) within 28 days of treatment. This nonspecific response was characterized by granularity of the spinal cord, bubbling of CNS and PNS myelin sheaths, and rapid breakdown of motor and sensory PNS fibers. In contrast, those explants treated with 10-400 ug/ml (non-cytotoxic levels) remained viable throughout the exposure period. However, after 38-49 days of treatment, intra-axonal rectilinear inclusions (Figure 4) developed in several cultures treated with 200-400 ug/ml MEK. These structures were often associated with axoplasmic granularity, although overt fiber degeneration was rarely seen. Electron microscopy revealed the granularity to correspond to foci of degraded axoplasm (Figure 5). Such foci were not noted in age-matched controls nor in n-hexane-treated cultures. Cultures treated with lower doses of MEK (10, 25, 50, 100 ug/ml) for similar periods and age-matched controls did not develop these foci (Table 2).

TABLE 2. DOSE RESPONSE FOR MEK^a

<u>MEK (ug/ml)</u>	<u>Response^b</u>	<u>Incidence of Effects</u>
10	NC	0/8
25	NC	0/8
50	NC	0/8
100	NC	0/8
200	RI (49)	3/8
300	RI (42)	5/8
400	RI (38)	5/8
600	Pancytotoxic	8/8

^a Explants (8 per dose) were treated for 49 days with 10-600 ug/ml of MEK dissolved in nutrient fluid.

^b MEK in doses of 600 ug/ml produced pancytotoxic effects whereas cultures treated with 10-100 ug/ml showed no morphological changes (NC) within the test period. Explants treated with 200-400 ug/ml developed rectilinear inclusions (RI) along the length of myelinated fibers. The time of their appearance was recorded in days.



Figure 3. Typical giant axonal swelling (arrow) seen in distal end of myelinated fiber in culture treated with n-hexane (250 ug/ml) for 42 days. Brightfield, living culture X1800.

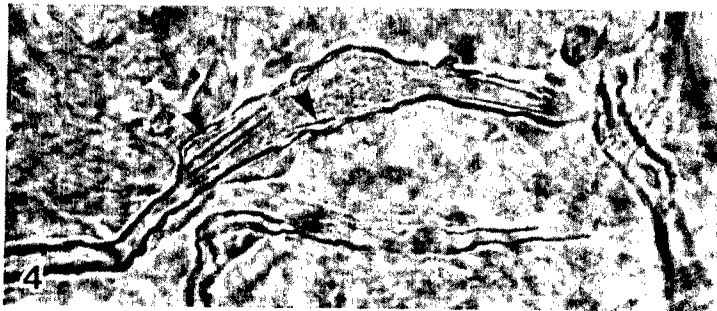


Figure 4. Intra-axonal rectilinear structures (arrows) are shown. Culture treated with MEK (300 ug/ml) for 42 days. Note axoplasmic granularity. Brightfield, living culture X1200.



Figure 5. Electron microscopy of rectilinear structures revealed them to be multiple foci of axoplasmic debris. Culture treated with MEK (300 ug/ml) for 42 days. Electron micrograph X12,000.

n-HEXANE: MEK-TREATED CULTURES

Table 3 reports the TTO of giant axonal swellings in the 10 groups of cultures treated with n-hexane:MEK mixtures. Irrespective of concentration, MEK (10-100 ug/ml) induced "NR" doses of n-hexane (25, 50 ug/ml) to produce typical giant axonal swellings within 21 days of exposure. These levels of MEK, in combination with neurotoxic doses of n-hexane (100, 250 ug/ml), accelerated the TTO of axonal pathology when compared with n-hexane treatment alone.

TABLE 3. DOSE RESPONSE FOR n-HEXANE:MEK^a

n-Hexane	MEK	0	10	25	50	100
0		NC	/	/	NC	NC
25		NC	/	/	11	22
50		NC	14	/	12	18
100		43	31	/	19	25
250		28	/	21	27	/

^a Explants (6 per dose) were treated for 4-8 weeks with n-hexane alone (0-250 ug/ml), MEK alone (0-100 ug/ml), or selected combinations thereof. The TTO for the appearance of axonal swellings to occur in 75% of the cultures is given in days. In all mixture-treated cultures, MEK was observed to enhance the TTO. NC: no change/: not tested.

Probit analysis (Table 4) confirmed this potentiation although there were insufficient or partial responses in some mixtures. Cox's model (Table 5) gives the corresponding coefficients for the various exposure combinations where trials were observed. These coefficients indicate relative effects of each material alone and in combinations as estimated from Cox's linear model for proportional hazards. These data indicate that the coefficient of the product of MEK and n-hexane levels was significantly different from zero when MEK and n-hexane were present in the model. Since this coefficient was highly significant, ($p < .001$) potentiation was indicated.

TABLE 4. PROBIT ANALYSIS OF MIXTURE-TREATED CULTURES^a

<u>MEK (ug/ml)</u>	<u>n-Hexane (ug/ml)</u>	<u>R/C^b</u>	<u>EC 50^c</u>
0	50	0/6	154 (108,335)
0	80	0/6	
0	100	2/6	
0	250	4/5	
50	0	0/6	<100 may be <25 ^d
50	25	6/6	
50	50	4/4	
50	100	9/12	
100	0	0/6	<25 ^d
100	25	6/6	
100	50	4/5	
100	100	5/5	

^a SAS, 1982.

^b R/C: the number of cultures responding after 28 days exposure/the total number of treated cultures.

^c 95% confidence intervals.

^d Insufficient or partial responses for probit analysis.

In addition to developing giant axonal swellings, some explants treated with n-hexane:MEK in mixtures of 25:50, 25:100, 50:10, 50:25, 50:50, 100:10, and 100:50 ug/ml, developed the rectilinear inclusions described previously. The timing of development and location of these inclusions were independent of the spatial-temporal characteristics of the n-hexane-induced axonal swellings.

DISCUSSION

This study describes a tissue culture model of MEK's potentiation of n-hexane neurotoxicity and again demonstrates the flexibility and potential usefulness of in vitro systems to evaluate neurotoxic chemicals. Two salient features of MEK's interaction with n-hexane are demonstrated in this study. First, MEK accelerates the onset of specific hexacarbon pathology, an observation that is supported by animal studies. Rats inhaling 9:1 mixtures of n-hexane (9000 ppm) and MEK (1000 ppm) for 8 h/d, 7 d/wk, displayed functional signs of neuropathy after 5 wks, whereas litter mates exposed to 10,000 ppm of n-hexane alone developed neuropathy after 8-10 wks (Altenkirch et al., 1978).

**TABLE 5. COX'S STATISTICAL ANALYSIS
OF MIXTURE TREATED CULTURES^a**

	MEK (ug/ml)				
	0	10	25	50	100
n-Hexane (ug/ml)					
0	1.0	0.9	0.9	0.7	0.6
25	1.1			1.1	1.1
50	1.2	1.3		1.6	2.1
100	1.5	1.7		3.4	8.0
250	2.6		9.4	34.0	

^a Cox's general linear model for proportional hazards (Cox, 1972; SAS, 1982) was used to examine the interaction of mixture-treated cultures. The table above gives predictive values of the coefficients of hazards where n-hexane, MEK and their multiplication products are included.

Similarly, in the organotypic culture system, specific neuropathologic changes appeared after 31 days of treatment to mixtures of n-hexane and MEK (100:10) whereas in the absence of MEK, explants treated with 100 ug/ml of n-hexane developed axonal swellings after 43 days of exposure. The second observation is that MEK lowers the neurotoxic threshold of n-hexane in vitro. Although "NR" levels of n-hexane (25, 50 ug/ml) failed to produce axonal swellings after 56 days of exposure, these levels when combined with MEK (10-100 ug/ml), produced the characteristic axonal pathology in 11-12 days. MEK's ability to induce "no-response" levels of n-hexane to express axonal degeneration is an entirely new experimental observation supported in part by clinical reports of solvent addicts in West Berlin. Between 1968 and 1975, these individuals chronically abused a commercial product containing 30% n-hexane without developing neuropathy. However, in October 1975, the n-hexane content of the product was lowered to 16%, and 11% MEK was added to the mixture. Within 6 months, 19 cases of severe hexacarbon neuropathy appeared, followed by 6 additional cases within the next 24 months (Altenkirch et al., 1977). It should be noted that in the reformulated product, the toluene fraction remained unchanged, the ethyl acetate fraction was decreased, and the benzene fraction was increased, raising the possibility that these ingredients might also have influenced

n-hexane's neurotoxicity. Other reports of occupationally-related solvent neuropathy involving commercial mixtures of MEK and n-hexane exist (Barone, 1973; Carapella, 1975; Gaultier and Rancruel, 1973; Goto et al., 1974). Unfortunately, quantitative and qualitative analysis of the product's ingredients were missing in these reports, preventing the identification of the culpable neurotoxic agent(s).

The mechanism(s) underlying MEK's influence on n-hexane neurotoxicity is still unknown. Experimental studies have demonstrated that both oral administration (Traiger and Bruckner, 1976) and subchronic inhalation exposure to MEK (Couri et al., 1977) induce rat hepatic microsomal activity. The combination of MEK with hexacarbons might alter the rate at which the parent compound(s) is converted to its neurotoxic metabolite(s) or influence the retention or elimination of the neurotoxic moiety. Another possibility is that MEK is able to displace or alter the binding affinity of neurotoxic hexacarbons to target tissues or enzymes, thus increasing the bioavailability of the toxicant. Another attractive explanation is that MEK's metabolic induction promotes a more rapid conversion of the parent hexacarbons n-hexane and MnBK to their putative primary neurotoxic metabolite 2,5-hexanedione (2,5-HD) which would result in a more rapid onset of neuropathy. Data from two studies, however, argue against this notion. Experiments have shown that animals continuously exposed to mixtures of MEK and MnBK display an elevated blood level of MnBK when compared to animals exposed to MnBK alone (Abdel-Rahman et al., 1976). Other experiments have demonstrated that 2,5-HD levels in the sciatic nerves of rats acutely exposed to n-hexane:MEK mixtures are actually lower than in nerves taken from animals exposed to equivalent concentrations of n-hexane alone (White and Bus, 1980). Note that this study involved single-dose administration of MEK rather than subchronic exposure, a dosing regimen which may not have produced the induction of microsomal activity needed to accelerate n-hexane's conversion to 2,5-HD. To address this possibility, levels of 2,5-HD should be measured in the nerves of animals subchronically exposed to n-hexane:MEK mixtures.

Finally, the appearance of intra-axonal, rectilinear inclusions in cultures treated with MEK alone and in combination with n-hexane merits discussion. Under electron microscopy, these structures were identified as foci of organelle degradation and appear to be a nonspecific response of the axon to toxic injury. Although these changes have not been reported in animals exposed subchronically to MEK (Saida et al., 1976; Spencer and Schaumburg, 1976; Altenkirch et al., 1977; Egan et al., 1980), their development may be ascribed to differences in bioavailability, pharmacokinetics, and biocompartmentation between the in vitro and in vivo systems. An ultrastructural examination of these structures is presented in a companion paper.

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MOLECULAR MECHANISMS OF n-HEXANE NEUROTOXICITY

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INTRODUCTION

The initial reports of neurotoxicity due to the "hexacarbon" solvents n-hexane and methyl n-butyl ketone (MnBK) (Yamada, 1967; Allen et al., 1975), and of their diketone metabolite 2,5-hexanedione (2,5-HD) (Spencer and Schaumburg, 1975) led to a substantial experimental effort to explore the biochemical and pathological characteristics of this syndrome. Such studies have been prompted not only because of the great potential for human exposure to these compounds (NIOSH, 1977) but also because of the desire to demonstrate a mechanism of action in common with other chemicals causing a similar neuropathy associated with axonal neurofilament accumulation (Figure 1). This type of neuropathy has been named "central-peripheral distal axonopathy" (CPDA) in an attempt to describe its most distinctive pathological features (Spencer and Schaumburg, 1976). In addition, the abnormal accumulation of neurofilaments induced by the neurotoxic diketones is not unlike that seen in certain naturally-occurring disorders such as childhood giant axonal neuropathy (Spencer and Schaumburg, 1977 a, b; Pena, 1982). They may therefore be useful tools for exploring the function of these cytoskeletal elements in normal and disease states.

A hypothetical molecular mechanism of action must ideally account for the structure/activity relationships, neurofilament accumulation, in vivo covalent protein binding, direct action on axonal components, and target organ specificity which are characteristic of the neuropathy caused by these compounds. Although this ideal has not yet been realized, substantial progress has been made toward elucidation of the crucial events leading to neuropathy. Ultimate success awaits exploration of the molecular interaction of the neurotoxic diketones with axonal components in vivo. The distinguishing features of n-hexane neuropathy are listed in Table 1.

NEUROFILAMENT ACCUMULATION

A pathological hallmark of n-hexane neuropathy is axonal swelling due to massive accumulation of 10 nm neurofilaments within the axoplasm of selected nerve fibers (Spencer and Schaumburg, 1977 a, b). These swellings are typically localized to the distal, non-terminal regions of long, large diameter myelinated nerve fibers, on the proximal side of nodes of Ranvier. Although

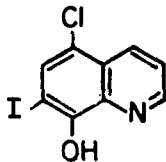
<u>Chemical</u>	<u>Structure</u>	<u>Site of Accumulation</u>
Acrylamide	$\text{CH}_2=\text{CH}-\text{C}(=\text{O})\text{NH}_2$	Distal
Carbon disulfide	$\text{S}=\text{C}=\text{S}$	Distal
Clioquinol		Distal
Dimethylaminopropionitrile	$\text{CH}_3-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{C}\equiv\text{N}$	Distal
Disulfiram	$\text{H}_5\text{C}_2-\text{N}(\text{C}_2\text{H}_5)-\text{C}(=\text{S})-\text{S}-\text{C}(=\text{S})-\text{N}(\text{C}_2\text{H}_5)-\text{C}_2\text{H}_5$	Distal
2,5-Hexanedione	$\text{CH}_3-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{C}(=\text{O})-\text{CH}_3$	Distal
Aluminum	Al^{3+}	Proximal
β,β' -iminodipropionitrile	$\text{HN}(\text{CH}_2-\text{CH}_2-\text{C}\equiv\text{N})_2$	Proximal

Figure 1.
Chemicals
causing
axonal
neurofilament
accumulation.

TABLE 1. CHARACTERISTICS OF DIKETONE NEUROPATHY

- Target organ specificity (nervous system, testis)
- Neurofilament accumulation in distal axon
- Structure/Activity relationships
- In vivo covalent protein binding
- Direct action on axonal components

the neurofilament accumulations are morphologically similar to those encountered after exposure to acrylamide, CS_2 , and β,β' -iminodipropionitrile, their location, severity, and distribution are somewhat different from those in these other neuropathies (Spencer and Schaumburg, 1974; Seppalainen and Haltia, 1980; Griffin et al., 1982). Disorientation of neurofilaments and

arrangement into whorled patterns is frequently observed, along with segregation of microtubules within a channel near the center of the axon. Studies have demonstrated the slowing of fast axoplasmic transport at areas of neurofilament accumulation along the nerve fiber (Griffin et al., 1977; Mendell and Sahenk, 1980). Electron microscopy and gel electrophoresis have not revealed any obvious alterations in neurofilaments from animals exposed to 2,5-HD, although this may reflect the inability to biochemically isolate regions of axonal swelling from more normal areas of nerve fiber (Spencer and Schaumburg, 1977a; DeCaprio et al., 1983). It is presently unknown whether altered neurofilament structure and subsequent aggregation represents a primary cause of the neurotoxic syndrome or is a secondary effect of some other critical biochemical lesion within the axon.

STRUCTURE/ACTIVITY RELATIONSHIPS

Spencer and co-workers (1978) demonstrated that of a variety of ketones and alcohols, only those with a γ -diketone structure (two carbon spacing between the carbonyl groups) or which could be metabolized to a γ -diketone were capable of inducing the characteristic neuropathy. Thus, 2,5-HD and 2,5-hexanediol were neurotoxic, while 2,4- and 2,3-HD were not. Later studies demonstrated the neurotoxicity of the γ -diketones 2,5-heptane-dione and 3,6-octanedione and of 5-nonanone, which was shown to be metabolized to MnBK and 2,5-HD (O'Donoghue and Krasavage, 1979; DiVincenzo et al., 1982). A recent report indicated that the γ -diketone 3,4-dimethyl-2,5-hexanedione produces a similar neuropathy (Anthony et al., 1983). All γ -diketones tested to date have demonstrated a neurotoxic potential, and this has led to several mechanistic hypotheses based on the unique chemical reactivity of these compounds. The structures of neurotoxic and non-neurotoxic diketones are illustrated in Figure 2.

STRUCTURE/ACTIVITY RELATIONSHIPS

IN DIKETONE NEUROPATHY

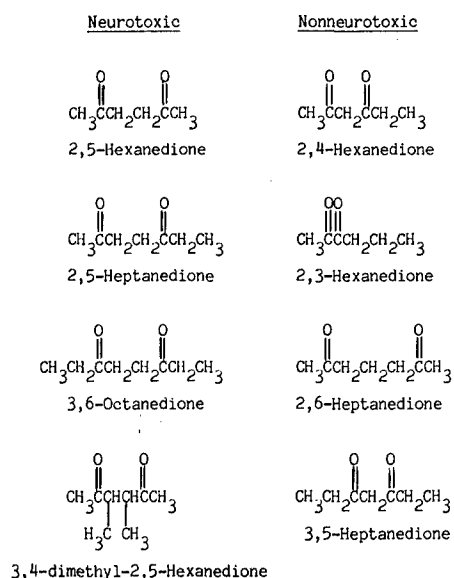


Figure 2. Structures of neurotoxic and non-neurotoxic diketones.

TARGET ORGAN SPECIFICITY

The γ -diketones exhibit an organ-specific toxicity in that only the nervous system and testicular germinal epithelium are affected (Spencer et al., 1980). Even at high dose levels with resultant wide tissue distribution of the compound, histologic or clinical evidence of damage to other organ systems is absent. Although body weight loss is frequently observed in experimental animals receiving 2,5-HD, Gillies et al. (1980a) have shown that this is primarily an effect of decreased food and water consumption. The phenomenon does not appear to be related to the mechanism of neurotoxicity, since neuropathy in hens exposed to 2,5-HD has been reported without accompanying body weight loss (DeCaprio et al., 1983). The target organ specificity of the γ -diketones has been attributed to the unique structural characteristics and energy requirements of the axon as compared with other tissues.

IN VIVO PROTEIN BINDING

DiVincenzo et al. (1977) reported the presence of protein-bound radiolabel in liver, kidney, and brain from rats receiving a single oral dose of $[1-^{14}\text{C}]\text{MnBK}$. Peak tissue protein levels occurred just after maximum blood levels of 2,5-HD were measured (8 hr vs. 6 hr after dosing), suggesting covalent reaction of metabolically-derived 2,5-HD with protein. Radiolabel in protein remained the same or decreased only slightly between 8 and 24 hr after dosing, again indicating the likelihood of covalent binding. Administration of $(^{14}\text{C})2,5\text{-HD}$ to rats via a single i.p. injection of 8 mg/kg resulted in residual label in both spinal cord and sciatic nerve 48 hr after dosing (Spencer et al., 1980). A later study reported high-affinity (covalent) binding of $(1,6-^{14}\text{C})2,5\text{-HD}$ to myelin and other subcellular fractions of sciatic nerve from rats after intraneural injection of the diketone (Sabri and Spencer, 1981). These results provide evidence for chemical reaction of 2,5-HD with tissue macromolecules in vivo and suggest that such binding may be critical to the evolution of diketone neuropathy.

DIRECT ACTION

The final characteristic related to the mechanism of the γ -diketones is their ability to act directly upon axonal components. Politis et al. (1980) demonstrated that application of 2,5-HD to intact exposed rat sciatic nerve resulted in typical axonal swellings and neurofilament accumulation localized to the exposed areas. Unexposed contralateral nerves displayed no alterations, and nerves exposed to non-neurotoxic 2,4-HD or 1,6-hexanediol developed only nonspecific fiber breakdown and Schwann cell necrosis. Experiments utilizing a structurally- and functionally-coupled explanted mouse spinal cord, dorsal root ganglia, and skeletal muscle tissue culture system were also able to reproduce γ -diketone neuropathy in vitro (Veronesi et al., 1983). Axonal swellings in motor nerve fibers were produced by nutrient fluid containing 2,5-HD (and metabolic precursors of

2,5-HD) but not by fluid with 2,4-HD. Recent studies have shown that direct intrafascicular injection of 2,5-HD into rat sciatic nerve induces a rapid (within 1 min) reorganization of axonal neurofilaments into disorganized bundles with exclusion of other axonal organelles (Griffin et al., 1983; Zagoren et al., 1983). Non-neurotoxic 2,4-HD again produced no alterations.

The above findings indicate that the γ -diketones induce neurofilament accumulation and distal axonopathy by a direct action on nerve fibers, possibly involving covalent binding to neurofilament or other axonal proteins. The results also suggest that non-neurotoxic diketones either do not react or react reversibly with axonal sites, bind to less critical sites, or form reaction products of lower toxic potential than the γ -diketones. Any proposed mechanism of action must be able to successfully incorporate these findings and to identify the actual molecular lesion in γ -diketone neuropathy.

PROPOSED MECHANISMS OF ACTION

Inhibition of Axonal Glycolysis: Spencer and Schaumburg (1978) made the initial suggestion that nerve degeneration in diketone neuropathy might be related to inhibition of glycolytic enzymes within the axon, particularly glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This hypothesis was based upon the work of Sabri and Ochs (1971, 1972), who demonstrated that inhibition of GAPDH by iodoacetate resulted in a reduction of ATP levels and a slowing of fast axoplasmic transport within affected nerve fibers. The inhibition was due to irreversible covalent reaction of the iodoacetate with critical active-site sulfhydryl groups of the enzyme. The non-sulfhydryl glycolytic enzyme lactate dehydrogenase (LDH) was not inhibited. In order to test the hypothesis that MnBK and/or 2,5-HD might act via irreversible modification of enzyme sulfhydryl groups, Sabri et al. (1979a) measured the activities of GAPDH and LDH after in vitro incubation with these compounds and with non-neurotoxic 1,6-hexanediol and acetone. Inhibition of GAPDH but not LDH was observed with MnBK and 2,5-HD, while the non-neurotoxic compounds had no effect on GAPDH. Pre-incubation of enzyme with neurotoxin was necessary for inhibition, and prior addition of dithiothreitol (DTT) was observed to protect the enzyme from inactivation. Similar effects were observed with intact rat sciatic nerve and rat brain homogenate exposed in vitro to MnBK or 2,5-HD. Additional studies (Sabri et al., 1979b) revealed a similar in vitro inhibition of the major glycolytic enzyme phosphofructokinase (PFK) by 2,5-HD but not by 2,4-HD. A 31% decrease in activity was also measured in brain homogenates from rats receiving 0.5% 2,5-HD in the drinking water for 10-12 weeks, although whether this represented a statistically significant reduction from control values was not reported. The authors concluded that these compounds react irreversibly with sulfhydryl moieties of both GAPDH and PFK, rendering the enzymes inactive. Spencer et al. (1979) postulated that the reduction in energy supplies due to glycolytic enzyme inhibition would produce a

slowing of axonal transport which would be particularly acute at regions of high energy demand, i.e. nodes of Ranvier. The compromised transport system would presumably only be able to supply replacement enzymes to the more proximal areas of the axon at the expense of distal regions. In addition, slowly transported neurofilaments would begin to accumulate at nodes of Ranvier, further aggravating the nutrient transport situation. The net result would be frank Wallerian-type nerve degeneration in areas distal to the neurofilament accumulations. The authors suggested that a similar mechanism might apply to other chemicals causing CPDA and neurofilament accumulation. This hypothesis is illustrated in Figure 3.

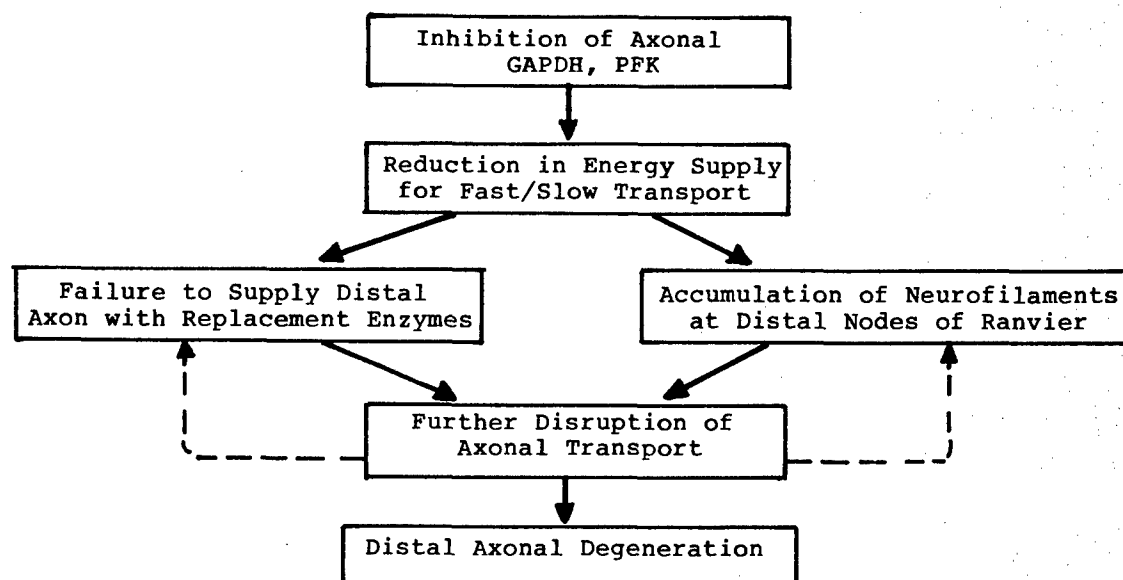


Figure 3. Axonal degeneration caused by inhibition of axonal glycolysis.

A variety of other studies have been performed in an attempt to support or refute this hypothesis. Howland et al. (1980) reported in vitro inhibition of enolase, another glycolytic sulfhydryl enzyme, by 2,5-HD. The inhibition differed from that of GAPDH and PFK since preincubation was not required and addition of DTT potentiated the inhibition. The authors concluded that the target organ specificity and lack of systemic effects of the γ -diketones might be due to selective inhibition of a specific neuronal isoenzyme of enolase. Couri and Nachtman (1979) demonstrated a decreased oxygen uptake in sciatic nerve from rats receiving 0.1 or 0.5% 2,5-HD in the drinking water. These data have been cited by several authors (Howland et al., 1980; Couri and Milks, 1982) as support for inhibition of glycolysis, although the decrease in oxygen uptake was neither dose-related nor correlated with clinical signs of neuropathy.

Griffiths et al. (1981) reported a decreased local glucose utilization in the superior colliculus of rats after 3 weeks exposure to 0.5% 2,5-HD in the drinking water, prior to the onset of clinically observable neuropathy. This effect was considered to reflect a lower energy demand due to impaired impulse conduction, rather than a primary disruption in energy supplies. Glycolytic enzyme activities were not reported in the study. Reasoning that inhibition of GAPDH should result in decreased supply of glycerol-3-phosphate and acetyl-CoA for lipogenesis, Gillies et al. (1981a) examined the incorporation of label from [^{14}C]glucose into sciatic nerve lipids from 2,5-HD treated rats. Synthesis of sterols was found to be depressed, while that of free fatty acids, triacylglycerols, and phospholipids was not different from that of pair-fed control animals. They concluded that while glycolysis was not inhibited in vivo, some defect in the lipogenic pathways leading from acetyl-CoA to sterols was present in γ -diketone exposed animals. A study of the effects of MnBK and its non-neurotoxic isomer isobutyl ketone on the sulfhydryl enzymes creatine kinase and adenylate kinase revealed that the non-neurotoxic compound was only slightly less inhibitory (Lapin et al., 1982). DTT protected against enzyme inhibition, although this protection was highly dependent on both temperature and concentration. The authors suggested that sulfhydryl groups were unlikely to be the critical target of MnBK and that enzyme inhibition alone could not account for the neurotoxic action. The effects of 2,5-HD on creatine and adenylate kinase activities were not reported in this study.

It is apparent that although the hypothesis of glycolytic inhibition in γ -diketone neuropathy is attractive in many ways, there are several major objections to the theory. Inhibition of GAPDH and PFK appears to require relatively high concentrations of diketone, with diketone:protein molar ratios approaching 10000:1. Such concentrations would probably seldom be encountered in nervous tissue, particularly after exposure to n-hexane, where conversion to 2,5-HD is relatively slow. In addition, sulfhydryl reactivity of the γ -diketones is weak compared to other classic inhibitors of this type (Graham and Abou-Donia, 1980). Conclusive evidence of in vivo enzyme inhibition during 2,5-HD intoxication is lacking, with only a single report of moderate (31%) inhibition of brain PFK in rats with severe paralysis and significant body weight loss (Sabri et al., 1979b). It is also difficult to account for neurofilament accumulation by inhibition of glycolysis. Although it has been shown that fast transport can be inhibited by disruption of glycolysis and lack of ATP (Sabri and Ochs, 1971, 1972), no similar energy requirement has been demonstrated for movement of neurofilaments in slow axonal transport. Indeed, the dynamics of neurofilament transport are obscure, and probably involve complex relationships with other cytoskeletal elements (Lasek and Hoffman, 1976). Perhaps the most significant objection to the theory is its failure to account for the extremely rapid reorganization of neurofilaments observed after intraneural injection of 2,5-HD (Griffin et al.,

1983; Zagoren et al., 1983). Such an effect suggests a direct interaction of the diketones with cytoskeletal structures rather than a slowly-developing disruption of energy supplies. Resolution of this question awaits studies on the spatial and temporal changes in energy status along individual nerve fibers during γ -diketone exposure.

PHYSICOCHEMICAL ALTERATION OF NEUROFILAMENTS

In 1980 it was suggested independently by two laboratories that the *in vivo* reaction site of 2,5-HD was the lysine ϵ -amino group rather than the cysteine sulfhydryl moiety of protein (DeCaprio and Weber, 1980; Graham et al., 1980). Both groups found that *in vitro* incubation of 2,5-HD with a variety of amines resulted in progressive irreversible reaction and the slow appearance of an orange chromophore in the incubation mixture. Based on an analogous reaction of malonaldehyde with amino acids (Chio and Tappel, 1969), Graham (1980) proposed the formation of fluorescent "conjugated Schiff bases" from the reaction of lysine amino groups and γ -diketones, structures which could conceivably account for the chromophore appearance. They further proposed that Schiff base formation could lead to intra- and intermolecular crosslinking between neurofilaments and eventual aggregation of these structures. The non-neurotoxicity of 2,4- and 3,5-diketones was attributed to their lack of water solubility and to their propensity for dimerization via internal hydrogen bonding. DeCaprio and Weber (1980, 1981) suggested instead that the reaction produced a 2,5-dimethylpyrrole moiety which underwent complex secondary reactions to yield chromophoric, oxidized polymers (Figure 4). This hypothesis was based upon well-known synthetic

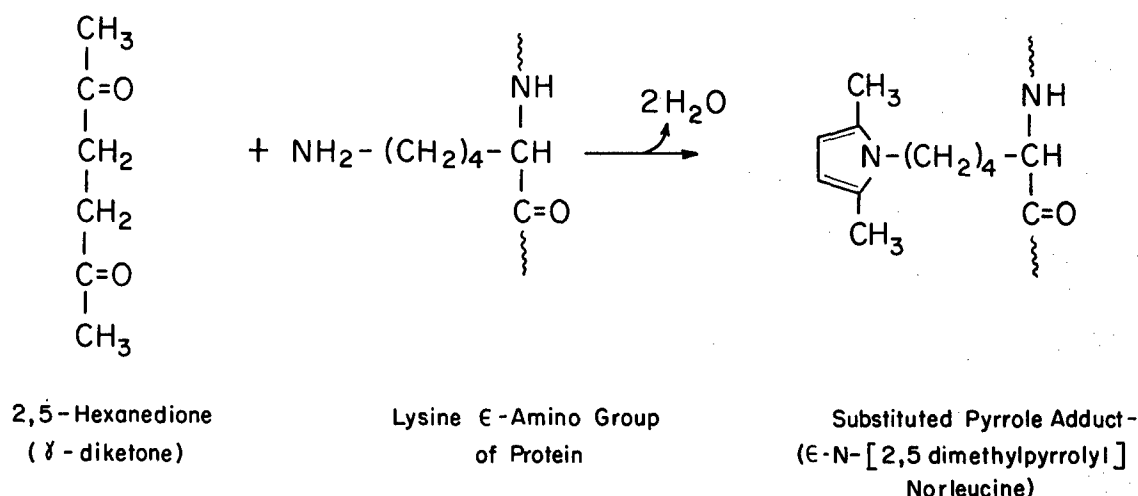


Figure 4. Reaction of 2,5-HD with protein lysine amino groups to form substituted pyrrole adducts.

routes for the 1,2,5-trisubstituted pyrroles (Waight, 1965; Patterson, 1976; Jones and Bean, 1977). Subsequent mass spectral and IR analyses confirmed the reaction product of the lysine ϵ -amino group and 2,5-HD as being the substituted pyrrole derivative ϵ -N-(2,5-dimethylpyrrolyl)norleucine (2,5-DMPN) (DeCaprio et al., 1982). The structure of the 2,5-HD-ethanolamine reaction product was similarly confirmed as a substituted pyrrole (Graham et al., 1982a). Incubation of other γ -diketones (2,5-heptanedione, 3,6-octanedione) with amines also yielded pyrrole products which underwent oxidation with time. Non-neurotoxic α - and β -diketones were found to react with amines, although the reversibility of reaction and structure of the products were not examined (DeCaprio et al., 1982). Gel electrophoretic analysis of protein treated in vitro with high concentrations of 2,5-HD (10000:1 diketone:lysine molar ratio) revealed progressive loss of lysine, formation of pyrrole adducts, polymerization, and color appearance. In contrast, incubation of protein at lower diketone:lysine molar ratios (1:1) resulted in loss of lysine and pyrrole adduct formation without polymerization. Reactivity of 2,5-HD with protein appeared to be linearly dependent upon lysine content, and at low diketone concentrations only the more basic proteins appeared reactive. These findings suggested a possible mechanism for selective in vivo protein binding by 2,5-HD. In vivo studies demonstrated the presence of the 2,5-DMPN residue in hydrolysates of serum protein from hens receiving 200 mg 2,5-HD/kg/day for 2 weeks (DeCaprio et al., 1982, 1983). A colorimetric assay specific for substituted pyrroles demonstrated widespread adduct formation in protein from serum, brain, liver, and kidney from hens exposed to 70 mg 2,5-HD/kg/day for up to 135 days, or to 200 mg/kg/day for up to 35 days. Gel electrophoresis revealed pyrrole adduct formation in specific proteins, including serum albumin, haptoglobin, and myelin basic protein. Binding of 2,5-HD with specific axonal cytoskeletal proteins could not be determined. Clearance of the adduct from tissue protein after cessation of exposure was also observed (DeCaprio et al., 1983).

The above findings confirm that the in vivo reaction product of 2,5-HD with protein lysine moieties is a 2,5-dimethylpyrrole adduct rather than a Schiff base derivative as previously suggested (Graham, 1980; Graham and Abou-Donia, 1980). Recent studies have demonstrated that animals receiving the 2,5-HD analog 3,4-dimethyl-2,5-HD (DMHD) rapidly develop a neuropathy characterized by neurofilament accumulations located in more proximal areas of axons than those encountered with 2,5-HD (Anthony and Graham, 1982). Based on these results, Graham et al. (1982a,b) proposed that in the initial stage, one of the carbonyl functions of 2,5-HD reacts reversibly with an amino group of neurofilament protein to yield an imine. This imine may then either "slowly" cyclize to the pyrrole or react with another amine function to yield a diimine in a competing reaction. The pyrrole ring would then be subject to oxidation and polymerization with other similar adducts on adjacent neurofilament protein

molecules, leading to intermolecular crosslinking and accumulation of these axonal cytoskeletal elements. They further suggested that the enhanced neurotoxic potential of DMHD was due to a more rapid formation of the pyrrole adduct, which would presumably allow crosslinking of neurofilaments to occur at an earlier point during their journey along the axon. The neurofilament accumulation would ultimately lead to a blockade of nutrient flow and frank nerve degeneration. The hypothesis has also been extended to account for the neurofilamentous neuropathies caused by acrylamide, CS₂, and IDPN, all of which are suggested to act via crosslinking (Anthony et al., 1983).

Several theoretical considerations and experimental findings argue against such a scheme. For example, the reaction mechanism of pyrrole formation is believed to proceed via a short-lived γ -amino ketone intermediate, rather than an unstable imine (Hazlewood et al., 1938) (Figure 5). Thus, competing reactions would not be available and pyrrole formation would be rapid and irreversible regardless of the structure of the diketone. Such imine formation has not been reported, even at very high diketone:amine molar ratios. In fact, 2,5-HD appears to react rapidly in vivo with protein amino groups, and distribution of pyrrole adduct is widespread after oral administration of the diketone (DeCaprio et al., 1983). In addition, the oxidation and polymerization phenomenon appears to be a light-catalyzed process requiring high concentrations of alkyl substituted pyrroles, conditions that would almost certainly not be encountered in vivo (Schofield, 1967; Jones and Bean, 1977). Pyrrole formation without secondary oxidation and crosslinking is characteristic of more toxicologically appropriate diketone:amine ratios. Covalent crosslinking of neurofilament protein has not been demonstrated, even in animals with severe γ -diketone-induced neuropathy (DeCaprio et al., 1983). Also, hydrolysis of such highly cross-linked neurofilament masses by proteases located in the nerve terminal would be expected to be difficult. In contrast, recent studies have demonstrated that masses which reach the terminal are readily removed (Cavanagh, 1982). It can also be hypothesized that if the distal vs. proximal distribution of neurofilament swellings is solely dependent upon the speed of formation and absolute level of pyrrole adduct, then simply increasing the effective axonal 2,5-HD concentration via increased dose should result in more proximally-located lesions. Such an effect has never been observed in diketone neuropathy. While such speculation is tempting, it may be premature to propose a similar mechanism for the other neurofilamentous neurotoxins, since they are not demonstrated crosslinking agents. For example, although CS₂ is known to react with spinal cord neurofilament protein in vivo, gel electrophoresis has not revealed any gross abnormality of these structures during CS₂ neuropathy (Savolainen et al., 1977).

As an alternative to a mechanism involving covalent crosslinking, it has been proposed that neurofilament accumulation may be a result of the increased hydrophobic interactions between these structures due to pyrrole adduct formation (DeCaprio et

al., 1982, 1983). The neurofilament proteins have inherently low water solubility (Liem, 1982) and thus the formation of even a few hydrophobic pyrrole adducts per molecule of protein might be sufficient to induce aggregation. Neurofilament masses that became lodged at nodal constrictions would block nutrient transport, while those that successfully reached the nerve terminal might be cleared effectively. Such a mechanism can also account

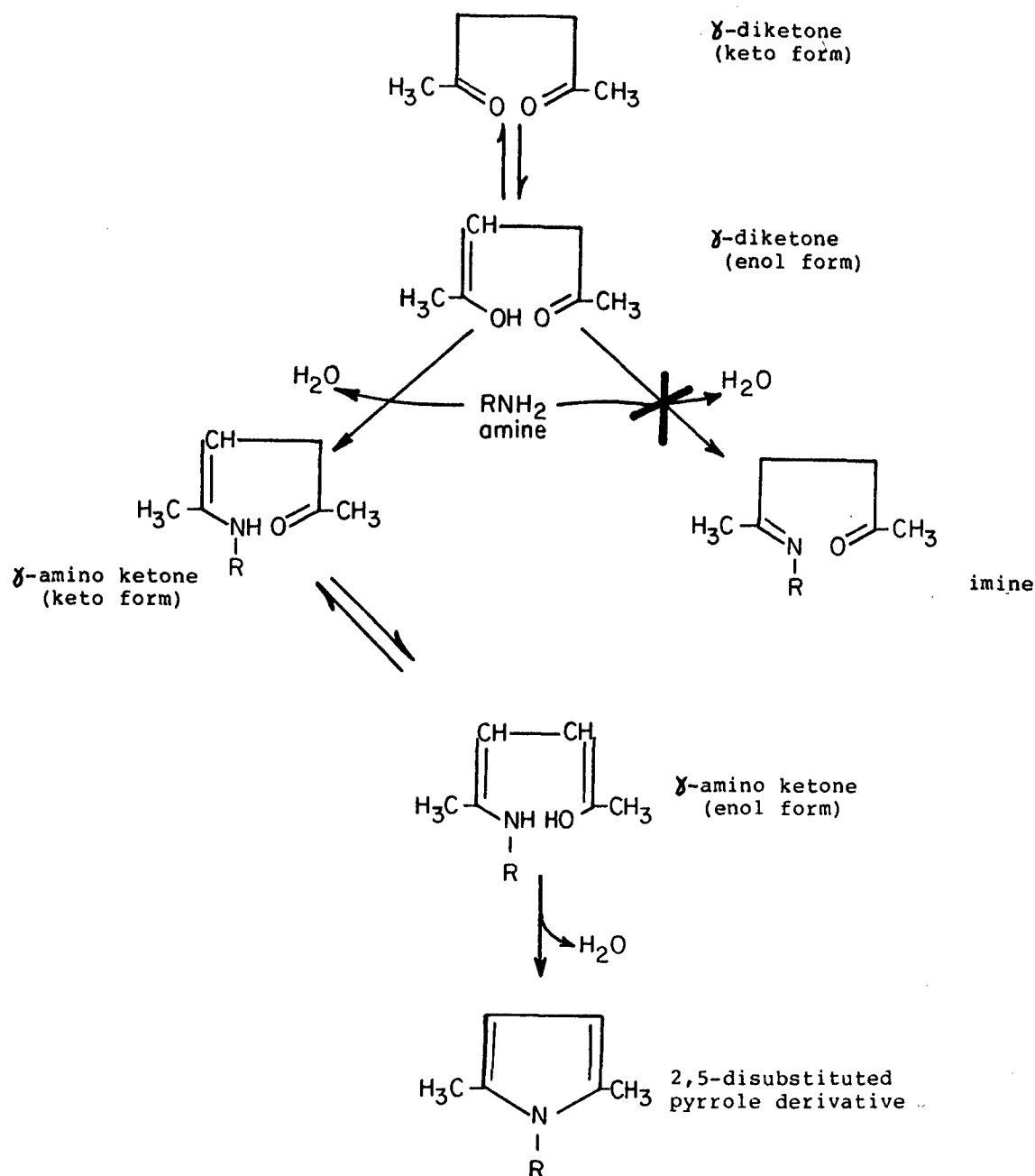


Figure 5. Reaction mechanism of pyrrole formation showing -amino ketone intermediate.

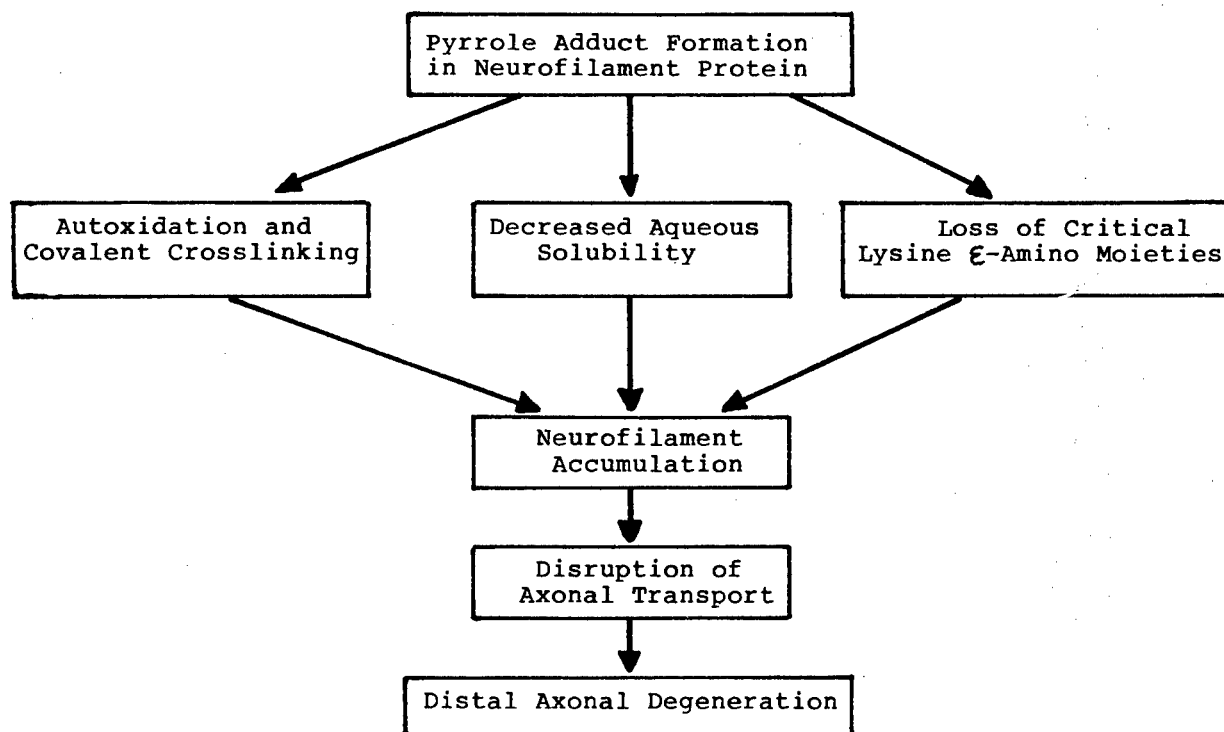


Figure 6. Hypotheses of axonal degeneration due to formation of pyrrole adducts in neurofilament protein.

for the more proximally-located swellings produced by DMHD, reaction of which would produce an extremely hydrophobic 2,3,4,5-tetramethylpyrrole adduct. Neurofilament protein modified in this fashion might conceivably lose solubility at an earlier point along the axon. Another possibility is that γ -diketone binding results in the loss of specific lysine amino groups that are essential to either the neurofilament transport process or to normal interaction between these structures and other nerve fiber components. The complexity of axonal cytoskeletal organization suggests that even minor alterations in this system could have profound neurotoxicological consequences (Lasek and Hoffman, 1976). The hypotheses of γ -diketone neuropathy involving a primary mechanism of pyrrole adduct formation in neurofilament protein are summarized in Figure 6. Elucidation of the molecular sites and levels of γ -diketone binding with axonal proteins *in vivo* will be necessary for confirmation of one of these hypotheses.

INHIBITION OF STEROLOGENESIS

Gillies et al. (1980a) reported decreased incorporation of [$1-^{14}\text{C}$]acetate into triacylglycerols, sterols, squalene, and ubiquinone in sciatic nerve of rats receiving 1% 2,5-HD in the drinking water for 6 weeks. Additional studies with [^{14}C]acetate and [^3H]mevalonolactone revealed a block in the pathway of ubiquinone synthesis between acetate and mevalonate, possibly at the level of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA

reductase) (Gillies et al., 1980b). The use of [^{14}C]leucine confirmed a specific inhibition of HMG-CoA reductase by 2,5-HD (Gillies et al., 1981b). The authors concluded that the inhibition of ubiquinone biosynthesis might contribute to 2,5-HD neuropathy via disruption of oxidative phosphorylation within the nerve.

It is difficult to account for all of the characteristics of γ -diketone neuropathy by a primary mechanism of HMG-CoA reductase inhibition and depletion of ubiquinone. Sterologogenesis from [^{14}C]acetate was not affected when rat sciatic nerves were incubated in vitro with 1 mM 2,5-HD, suggesting that the compound did not directly interact with the enzyme (Gillies et al., 1980b). In addition, Spencer et al. (1981) demonstrated that administration of sodium dichloroacetate, a known inhibitor of HMG CoA-reductase, could not produce a neuropathy similar to that from 2,5-HD. It is likely that this phenomenon represents a secondary response of the nerve fiber rather than a primary mechanism of neuropathy, although it may be important in testicular atrophy caused by 2,5-HD (Gillies et al., 1981b).

OTHER PROPOSED MECHANISMS

Couri and Nachtman (1979) reported biophysical changes in sciatic nerve homogenates and purified myelin preparations from rats receiving 0.1% or 0.5% 2,5-HD in the drinking water for two months. Arrhenius plots revealed a lack of normal temperature phase transitions in nerve homogenates from treated animals, and these changes were attributed to alterations in myelin lipid bilayers. Myelin membrane microviscosity was also decreased in animals at the 0.5% 2,5-HD level. The authors concluded that the membrane effects were not attributable to direct interaction of the diketone with membrane components, since addition of 2,5-HD to control nerve homogenates could not reproduce the changes. Clinical signs of neuropathy were not observed in animals at the 0.1% dose level, suggesting that the membrane effects preceded pathologic changes in the nerve fiber. Additional studies revealed that similar alterations were present in red cell membranes from 2,5-HD treated rats, and that such changes resembled those seen during Wallerian degeneration following nerve section (Nachtman and Couri, 1981). It has been proposed that these membrane effects may be related to the pathogenesis of myelin changes or to the mechanism of axonal degeneration in γ -diketone neuropathy (Couri and Milks, 1982).

Other mechanisms have also been proposed without any direct experimental evidence. Based on structural similarities to the potassium ionophore valinomycin, it was suggested that 2,5-HD might act to disrupt axonal membrane ion balance (Spencer et al., 1980). Schoental and Cavanagh (1977) speculated that the γ -diketones might simultaneously react with a thiol group of a thiamine-dependent enzyme (such as pyruvate decarboxylase) and with an amino group of thiamine itself. The covalent bridge thus formed would presumably destroy the catalytic activity of the

enzyme. Other proposed mechanisms include chelation of mitochondrial calcium and acetylcholinesterase inhibition (Couri and Milks, 1982). None of these hypotheses can account for all of the pathologic and biochemical characteristics of γ -diketone neuropathy.

FUTURE RESEARCH DIRECTIONS

Although substantial progress has been made since the initial reports of n-hexane and MnBK neurotoxicity, the molecular mechanism of action of these compounds remains elusive. Experimental evidence suggests that the mechanism almost certainly involves direct, probably covalent, interactions of 2,5-HD with lysine amino groups of neurofilament or other axonal proteins. Qualitative and quantitative evaluation of this binding during in vivo exposure to 2,5-HD is a prerequisite to further progress in this area. The difficulty of the task is evident, considering that a large proportion of absorbed 2,5-HD may become sequestered in non-neural protein "sinks" such as serum and myelin protein and thus never reach the axon (DeCaprio et al., 1983). In fact, the actual average level of pyrrole adduct formation in rat neurofilament protein after 9 weeks of oral exposure to 0.5% 2,5-HD may be less than a single altered lysine moiety per molecule of protein (DeCaprio, unpublished results). The changes in neurofilament protein structure and function as a result of such low level binding must be subtle indeed, and will be difficult to elucidate. Fortunately, the significant progress which has been made in purification and characterization of the neurofilament and other axonal proteins should make further advances possible.

A related objective is assessment of the proximal to distal distribution of both γ -diketone and altered protein in nerve fibers from treated animals. Since 2,5-HD penetrates lipid bilayers poorly (Couri and Nachtman, 1979), it is likely that the myelin sheath provides a substantial barrier to diffusion of diketone into the axoplasm and that the major sites of entry are via the nerve cell body, the nodes of Ranvier, and the nerve terminals. The generally distal location of neurofilament accumulations is difficult to account for based solely upon equal distribution of the diketone along the axon and progressive modification of neurofilament protein during axonal transport. If that were the case, increasing the dose of γ -diketone would be expected to induce more proximal accumulations, rather than merely speeding up the appearance of distal swellings as is actually seen. This observation can be accounted for in two ways. In the first scheme, diketone might preferentially enter the axoplasm via the nerve terminals and subsequently react with neurofilaments in the distal axon only. Since there probably is a large excess of potential amine binding sites as compared with diketone molecules, raising the dose would increase the number of adducts formed in distal neurofilaments only, therefore inducing their more rapid aggregation. The alternative to this scheme suggests

that there is a fundamental difference in either the neurofilaments themselves or the slow transport process between the proximal and distal axon such that the distal axon displays a greater vulnerability to the neurotoxin. No such spatial variation has yet been demonstrated. It is apparent from such speculation that determination of the sites of uptake and characteristic distribution of γ -diketones within the axon is vital to further understanding of their mechanism of action.

Many other mechanistic aspects of this neuropathy also need to be addressed. For example, non-neurotoxic diketones such as 2,4-HD appear to have some affinity for lysine ϵ -amino groups in vitro (DeCaprio et al., 1982). If these compounds display similar in vivo reactivity, then the question arises as to whether they react with the same sites as the γ -diketones. Such a finding would suggest that formation of a specific reaction product rather than simple loss of a particular lysine moiety might be crucial to induction of the syndrome. As mentioned previously, the status of energy production and usage along the nerve fiber needs to be explored, since several lines of evidence indicate that this parameter is altered in γ -diketone neuropathy. It is likely that experiments of this nature will serve to illuminate many aspects of this neuropathy and would allow progress toward elucidating a possible common mechanism for the γ -diketones and other neurofilamentous neurotoxins.

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PATHOLOGY AND AXONAL TRANSPORT IN HEXACARBON NEUROPATHIES

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INTRODUCTION

The hexacarbons came to attention because of their profound neurotoxic clinical effects (Allen et al., 1975; Mendell et al., 1974). These initial studies laid the groundwork for their present role as important experimental models. Chronic low dose hexacarbon exposure in a variety of species including rats, cats, and chickens (Mendell et al., 1974; Saida et al., 1976) results initially in axonal swellings containing normal appearing 10 nm filaments. These neurofilamentous swellings are found on the proximal side of the nodes of Ranvier in distal non-terminal regions of nerve fibers in both the peripheral and central nervous systems (central-peripheral distal axonopathy) (Spencer et al., 1977). With continuous exposure, axonal degeneration begins in the axon distal to the swellings. These pathologic changes can be correlated with a gradual impairment of fast anterograde axoplasmic transport which we believe plays an important role in axonal breakdown. Furthermore, recent studies (Sahenk et al., 1981) also show an abnormality in the transport of retrograde organelles indicating that the defect involves the bidirectional transport of membranous organelles.

AXONAL TRANSPORT STUDIES IN HEXACARBON NEUROPATHIES

Slow Transport

The most apparent pathologic changes in the axon following hexacarbon exposure is the accumulation of neurofilaments. Since neurofilaments make up an important component of slow transport moving at 0.2 - 1.0 mm per day, in close association with microtubules (Black et al., 1980), their abundance in the axon suggests an abnormality in this phase of transport. Despite these findings, the mechanism(s) of neurofilamentous increase in the axon is poorly understood. Several possibilities have been proposed. Anthony and co-workers (1982) have postulated that covalent cross-linking of neurofilaments induced by the hexacarbons results in their accumulation. A second possibility, proposed by Monaco et al. (1983), is an accelerated transport of neurofilaments which they observed in the visual system of 2,5 hexanedione intoxicated rats. Other possibilities include increased local

proliferation or an alteration in degradation of neurofilaments. Further work, however, will be necessary to identify the exact mechanism(s) of neurofilamentous increase.

Fast Transport

As opposed to the rather poor understanding of the effects of hexacarbon on the slow transport system, there is convincing evidence that the rate of fast anterograde transport is reduced in the hexacarbon neuropathies (Mendell et al., 1977). Following the injection of $^3\text{[H]}$ leucine into the spinal cord or dorsal root ganglion of intoxicated rats, the mean rate of fast anterograde transport is progressively slowed and can be correlated with the clinical and pathologic severity of the neuropathy (Mendell et al., 1977). Furthermore, careful analysis of the data using two time interval studies (Mendell et al., 1977) showed that the defect was more pronounced distally where there were more abundant axonal swellings. Using autoradiography, Griffin et al. (1977) also observed impaired transport of labelled membranous organelles through these swollen axons.

More recently using 2,5 hexanedione intoxicated rats, we were able to demonstrate that not only was anterograde transport impaired, but also the return of retrogradely radiolabelled materials from the distal nerve terminals was delayed (Sahenk et al., 1981). These studies were done by calculating the amount of returning activity to a distal collection ligature over a 5 to 35 hour period using a method described by Bisby (1977).

Collectively, these studies provide evidence that the movement of rapidly transported materials is impaired in the hexacarbon exposed axon especially in sites related to the neurofilamentous axonal swellings. Furthermore, the transport defect involves the bidirectional movement of organelles in the axon.

MORPHOLOGIC ALTERATIONS RELATED TO AXONAL TRANSPORT

Abnormalities

A more complete understanding of the significance of the transport abnormalities in the hexacarbon neuropathies has emerged through careful studies correlating transport abnormalities with structural changes. The earliest structural change in hexacarbon-exposed axon is a reorganization of the cytoskeleton whereby the microtubules cluster into groups seemingly segregated by or from neurofilaments. This abnormality can be dramatically demonstrated within 2 hours following the local application of 2,5 hexanedione beneath the perineurium of the rat sciatic nerve (Griffin et al., 1983). In these studies, there is a distinctive cytoskeletal change whereby microtubules are collected into a central channel with neurofilaments segregated in a subaxolemmal ring. These studies raise the strong possibility of a direct local effect of the hexacarbons segregating neurofilaments from

the rest of the axonal cytoskeleton. This segregation could impair the proximal-to-distal transport of neurofilaments resulting in the formation of neurofilamentous axonal swellings. A similar cytoskeletal change is also noted following the systemic intoxication of rats with 2,5 hexanedione. In these chronically exposed axons the channels are seen in close association with membranous organelles, small vesicles, and elongated tubulovesicular structures which are thought to be the major moving components of the fast transport system (Sahenk et al., 1983). Figure 1 shows one of these channels in a longitudinal plane of section in an axon stained with diaminobenzidine potassium ferrocyanide (Sahenk et al., 1983) to enhance visualization of the axonal membranous organelles including smooth endoplasmic reticulum and free vesicles. It is important to note that the channels, in addition to microtubules, also possess the longitudinal tubules of smooth endoplasmic reticulum indicating a close functional relationship of these axonal elements in transport process.

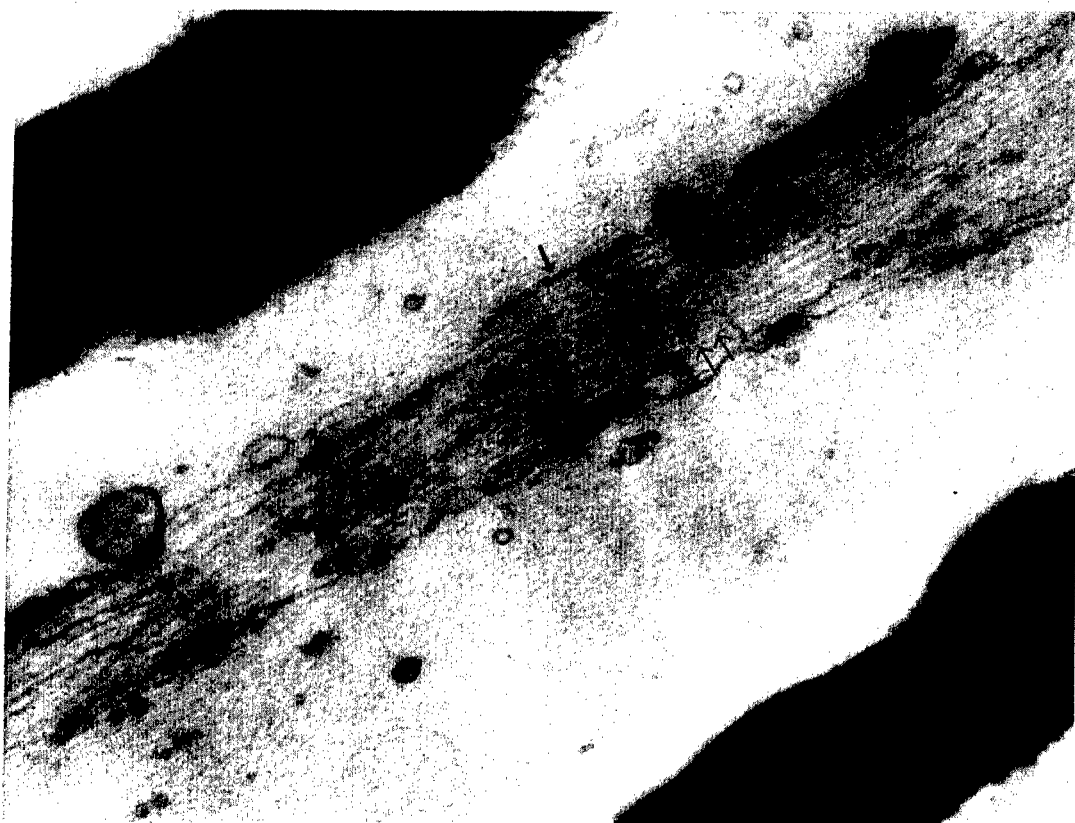


Figure 1. 2,5 hexanedione treated axon from the posterior tibial nerve showing the presence of clustering of microtubules (double arrows) in close association with smooth endoplasmic reticulum (single arrow) forming into a channel. Note the vesicles in close vicinity to those longitudinal elements; 0.3 μ m thick section, diaminobenzidine potassium ferrocyanide staining. x 14,000.

The association of these microtubule channels with membranous organelles (Sahenk et al., 1983; Papasozomenos et al., 1982) suggests that these channels provide a passage for fast transported materials within the axon. This is further supported by studies in another neurofilamentous neuropathy induced by B,B'-iminodipropionitrile (IDPN) where similar channels are also observed. In the IDPN model, autoradiographic studies by Papasozomenos et al. (1982) also demonstrated the normal passage of radiolabelled materials through these microtubule channels.

During continuous exposure to hexacarbons, there is a further increase in the density of neurofilaments, especially in the swollen axons. In these swellings the neurofilaments become maloriented and there is a further disruption of the linear orientation of the microtubule channels (Figure 2). This appears to impair the continuity of these channels so that membranous organelle transport within the axon is severely altered. This is well demonstrated using the diaminobenzidine potassium ferrocyanide stained axons in combination with high resolution electron microscopy (Figure 3), where careful quantitation showed a striking increase in the number of vesicles as well as fragmentation of the smooth endoplasmic reticulum (Sahenk et al., 1983).



Figure 2. Cross-section of a neurofilamentous swelling from methyl n-butyl ketone treated rat showing severe cytoskeletal disarray with maloriented microtubules (arrows) and neurofilaments. x 18,000.

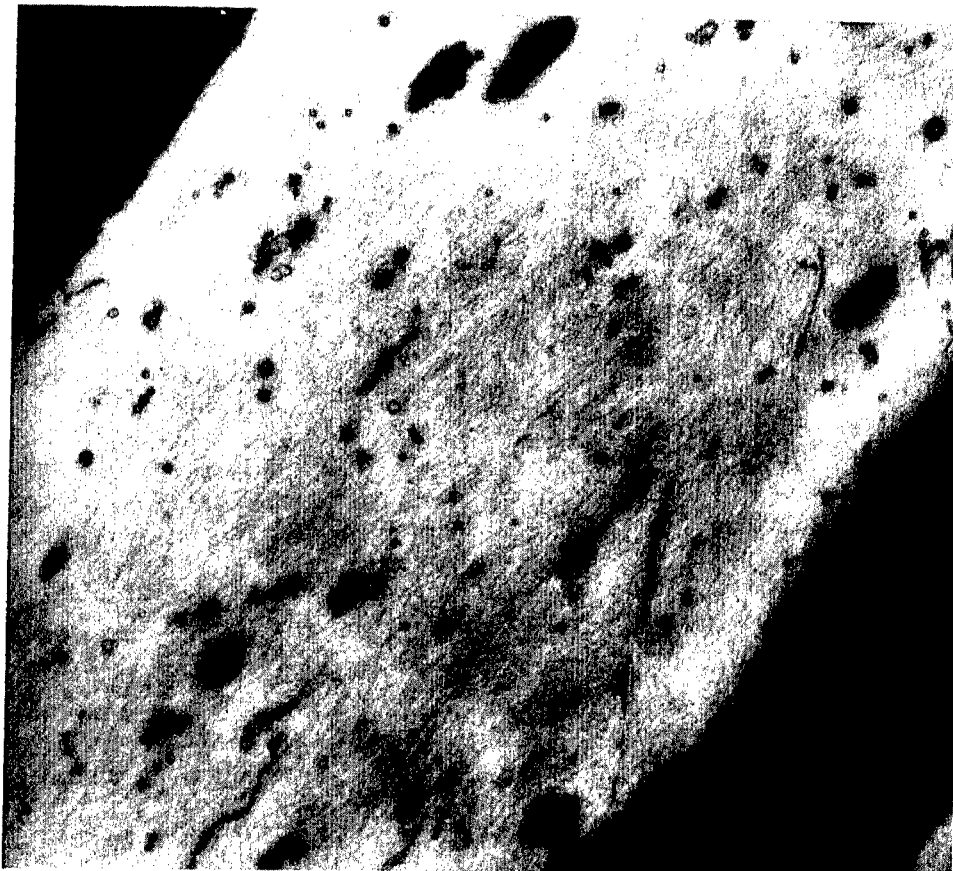


Figure 3. Longitudinal section from swollen 2,5 hexanedione treated axon showing significantly increased number of free vesicles, disorganization, and fragmentation of smooth endoplasmic reticulum. 0.3 μ m thick section diaminobenzidine potassium ferrocyanide staining. x 13,950.

The combination of these morphologic studies in association with previously observed transport abnormalities also have implications in regard to the morphologic substrate for fast anterograde transport. In fact, these studies suggest that microtubules (despite prior evidence to the contrary references Byers, 1974; Hansson et al., 1971; and Sjöstrand et al., 1970), smooth endoplasmic reticulum, and vesicles are important interacting components of fast anterograde transport system. As to which membranous component actually provides the movement for transport, a number of studies strongly indicate that vesicles and the elongated tubulovesicular structures are the moving components carrying the membrane associated proteins and glycoproteins (Allen et al., 1982; Brady et al., 1982).

With regard to the observed retrograde transport abnormality (Sahenk et al., 1981), we have also identified morphologic changes corroborating this finding. When one carefully examines the swollen axons in the hexacarbon neuropathies, granular, large particles can be seen to accumulate in the axon particularly on

the distal side of the paranodal swellings (Figure 4). These coarse, granular particles correspond to the group of membranous organelles composed of multivesicular bodies and large vesicles which travel retrograde in the axons and are thought to contain lysosomal activity. Through a special immunocytochemical staining technique, we found that these coarse, granular particles contain cathepsin D activity suggesting a lysosomal content (Sahenk et al., in press).

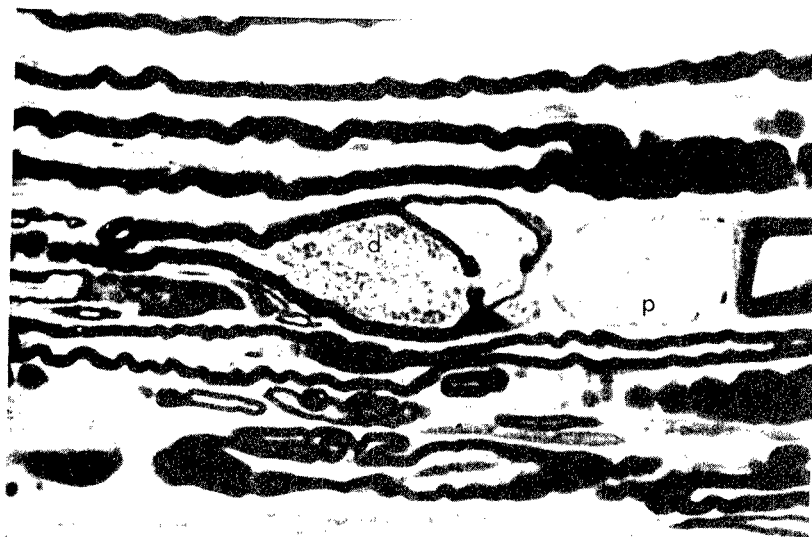


Figure 4. Longitudinal section of swollen axon in 2,5 hexanedione exposed posterior tibial nerve showing the accumulation of coarse, granular, large particles carried by retrograde transport in the distal paranodal segment. The distal (d) and proximal (p) paranodal segments are labelled. 1 μ m thick section, toluidine blue staining. x 1,200.

Conclusions

These studies lead us to propose the following hypothesis: The earliest change in the axon following hexacarbon exposure is an alteration in the cytoskeleton, resulting in clusters of microtubule channels segregated from neurofilaments. Initially, fast anterograde transport continues through these functioning channels. With continued exposure, the cytoskeleton becomes chaotic, and pathways for moving organelles are impaired, affecting both anterograde and retrograde transport. The severe disruption in axoplasmic transport seems to play a key role in axonal degeneration, at least in part related to the failure of delivery of important materials to the distal axon. Furthermore, the accumulation of lysosomal material, carried by retrograde transport, may play a role in the initiation and/or acceleration of the intraaxonal digestive process which is part of nerve fiber degeneration.

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SESSION II

DELAYED NEURODEGENERATIVE EFFECTS OF ORGANOPHOSPHOROUS COMPOUNDS IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

Chairman

**Rudy J. Richardson, Sc.D.
The University of Michigan
Ann Arbor, Michigan**

**ORGANOPHOSPHORUS-INDUCED DELAYED NEUROTOXICITY:
SYNDROME AND EXPERIMENTAL MODELS**

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INTRODUCTION

Several organophosphorus esters inhibit acetylcholinesterase and produce acute neurotoxicity that is transient and ameliorated with anticholinergic drugs. In addition, certain organophosphorus esters produce delayed neurotoxicity evidenced as paresis or paralysis having a delayed onset. Nerve destruction, or more specifically symmetrical central-peripheral distal axonopathy, accompanies organophosphate-induced delayed neurotoxicity (OPIDN).

Humans and certain experimental animals (e.g. hens, cats, and cattle) are highly susceptible to OPIDN and often show clinical impairment after a single exposure. Other experimental animals, especially rodents, are relatively resistant to OPIDN, and multiple treatments produce only mild evidence of neurotoxicity.

This study was conducted to compare neurotoxicity in hens and rats produced by tri-o-cresyl phosphate (TOCP) delayed neurotoxicant. A metabolic inhibitor, piperonyl butoxide, was co-administered with TOCP in rats in an attempt to reduce their resistance to OPIDN.

MATERIALS AND METHODS

ANIMALS

Adult White Leghorn hens in full egg production, 12-14 months old, were used. They were housed individually in a laboratory environment with controlled temperature (16-26°C) and lighting schedules (12 hour light, 12 hour dark). They had continuous access to food (Layena, Ralston Purina Co., St. Louis, MO) and water, except 16-24 hours before treatment.

Adult male CD® rats (Charles River Laboratory) were also used and housed under similar laboratory conditions. They were fed Purina Certified Rat Chow except when fasted prior to treatments.

CHEMICALS

Practical grade tri-o-tolyl phosphate (TOTP or TOCP, Eastman Organic Chemicals, Rochester, NY), and corn oil (Mazola, CPC International, Inc., Englewood Cliffs, NJ) were the test compounds used. All other chemicals were reagent grade.

EXPERIMENTAL DESIGN

Two groups were used to assess the delayed neurotoxic potential of TOCP in adult hens. Normal body weight, food consumption, and walking behavior (Sprague et al., 1980) were determined for a 2-week observation period. Each group was then treated as shown below, and the treatments were repeated 3 weeks later. Hens were killed 3 weeks after the second treatment and nerve tissues obtained. Body weight and food consumption were measured every 3-4 days, and walking behavior was measured weekly.

<u>Treatment</u>	<u>n</u>	<u>Dose</u>	<u>Vehicle</u>
Corn oil	10	10 ml/kg	Neat
TOCP	10	500 mg/kg	Corn oil

Four groups of rats were used to assess the delayed neurotoxicity of TOCP in rats. Each group was given 2 treatments. An i.p. injection (saline or piperonyl butoxide) followed by TOCP (p.o.). Body weights and rotarod performance were measured at weekly intervals and all animals were terminated 4 weeks after the TOCP treatment.

<u>Group</u>	<u>n</u>	<u>Treatments (mg/kg)</u>		<u>TOCP</u>
		<u>Piperonyl</u>	<u>Butoxide</u>	
1	10	0		0
2	10	400		500
3	10	0		0
4	10	400		500

Surviving or moribund hens were anesthetized with sodium pentobarbital (180 mg/kg ip or 90 mg/kg iv), exsanguinated, and perfused systemically (cardiac infusion) with ice-cold 10% neutral buffered Formalin NBF. Perfused hens were eviscerated and immediately submerged in Formalin for 24 hours, after which the intact brain, spinal cord, and distal portions of right and left sciatic nerves were removed. Tissues were stored for an additional 48 hours in Formalin and then trimmed for processing. Histologic specimens examined by light microscopy included medulla cut transversely; longitudinal and transverse sections of

midcervical, midthoracic, and lumbosacral spinal cord; and longitudinal and transverse sections of right and left sciatic nerves and the two major branches. Sections of each specimen were stained with Luxol Fast Blue and counterstained with periodic acid-Schiff and then hematoxylin and eosin (LFB/PAS/HE).

Rats were anesthetized with ether and perfused systemically with NBF. Cervical spinal cord and right and left sciatic nerve sections from rats treated with saline and corn oil and saline and TOCP were examined microscopically. Only cervical cord cross-sections were examined from rats treated with piperonyl butoxide and TOCP.

DATA ANALYSIS

Mean body weights and 24-hour food consumption were compared by Dunnetts Test (Tallarida and Murray, 1981).

RESULTS

DELAYED NEUROTOXICITY IN HENS

Hens treated with TOCP showed no abnormal signs until 10-14 days after the first treatment. At that time, they showed mild incoordination that was obvious while they moved about in their cages. Impairment was also obvious in the walking behavior measured 14 days after the first treatment. The mean score for TOCP-treated hens was 8 compared to a score of 0 for corn oil-treated hens. The mean score continued to increase to 12 when the last measurement was made 7 days prior to termination. Other general signs of toxicity were relatively mild. Food consumption was reduced for 2-4 days after each treatment in both groups (corn oil- and TOCP-treated). TOCP-treated hens showed a 17% loss of body weight during the study while body weights for corn oil-treated hens remained stable.

Some mild histopathologic changes, unrelated to treatment, were noted in brain, spinal cord, and sciatic nerve sections of corn oil-treated hens (Table 1). These changes were probably representative of subclinical Marek's disease, as might be expected in commercial poultry (Calnek and Witter, 1978). The most common changes in the central nervous system (CNS) were a moderate incidence of perivascular cuffing and focal glial cell proliferations. The most common background change in the peripheral nervous system (PNS) was the occurrence of lymphocytic foci in the perineurium of sciatic nerves.

**TABLE 1. INCIDENCES OF HISTOPATHOLOGIC CHANGES
IN HENS TREATED WITH CORN OIL OR TOCP**

<u>Tissue</u>	<u>Histologic change</u>	<u>Treatment</u>	
		<u>Corn oil^a</u>	<u>TOCP^b</u>
Brain	Axonal degeneration in cerebellar peduncles	0/10 ^c	10/10
	Neuronal swelling with chromatolysis	0/10	0/10
Cervical spinal cord	Axonal degeneration in dorsal tracts	0/10	10/10
	Focal gliosis	5/10	9/10
Thoracic spinal cord	Axonal degeneration in ventral and lateral tracts	1/10	10/10
	Focal gliosis	6/10	10/10
Sacrolumbar spinal cord	Axonal degeneration in ventromedial tracts	1/10	7/10
	Focal gliosis	5/10	7/10
Sciatic	Bilateral nerve fiber degeneration	0/10	9/10
	Axonal swelling	2/10	9/10
	Lymphocytic foci	6/10	5/10

^a10 ml/kg x 2

^b500 mg/kg x 2

^cValues are number of hens showing the specified change divided by the number of hens in the group

CNS and PNS specimens from TOCP-treated hens exhibited background changes similar to those in the corn oil group. However, hens in the TOCP group showed additional changes, including minimal to slight axonal degeneration in cerebellar peduncles and minimal to moderate axonal degeneration in dorsal funiculi, ventral and lateral funiculi, and ventromedial funiculi of cervical, thoracic and sacrolumbar spinal cord, respectively. Axonal damage was frequently accompanied by reactive focal gliosis. In the PNS, 9 of the 10 hens showed bilateral sciatic nerve fiber degeneration (i.e., degeneration in both right and left nerves) which was most severe in the distal branches.

DELAYED NEUROTOXICITY IN RATS

No rats showed evidence of hindlimb weakness or paralysis throughout the 4-week observation period after TOCP administration (with and without piperonyl butoxide pretreatment), and weekly rotarod performance was not affected. There were scattered failures on the rotarod but none of these indicated a pattern of progressive motor impairment. Initial and terminal body weights (Table 2) for treated rats were not markedly reduced.

TABLE 2. BODY WEIGHTS OF TREATED RATS

<u>Treatment</u>	<u>Body Weight, gms</u>	
	<u>Initial</u>	<u>Terminal</u>
saline & corn oil	198.7 \pm 14.2 (10) ^a	410.1 \pm 3.7 (10)
piperonyl butoxide & corn oil	201.0 \pm 11.9 (10)	410.1 \pm 26.2 (10)
saline & TOCP	189.3 \pm 16.3 (10)	377.4 \pm 31.2 (10)
piperonyl butoxide & TOCP	197.8 \pm 13.6 (10)	400.7 \pm 23.2 (10)

^aMean \pm standard deviation (N)

Nerve specimens from rats treated with saline and corn oil were normal. No bilateral changes were noted in peripheral nerves and cervical cords were normal. Peripheral nerves were also normal in rats treated with saline and TOCP. However, swollen eosinophilic axons within the tractus gracilis were noted in rats treated with saline and TOCP or piperonyl butoxide and TOCP. The incidence was higher in rats pretreated with piperonyl butoxide (70%) than in those pretreated with saline (50%). Most changes in the former group were rated as moderate to severe while the latter changes were primarily mild.

DISCUSSION

This study was designed to compare OPIDN in rats and hens after a single, massive dose. Two other groups have recently examined OPIDN in rats. Lasker and coworkers (1982) studied the comparative microsomal metabolism of the delayed neurotoxicant, O-ethyl O-4-nitrophenyl phenyl phosphonothioate (EPN) in 2 species with differing sensitivity to OPIDN. They found that rat liver microsomes exhibited 3-20 fold greater rates for EPN metabolism than liver microsomes from a sensitive species (hen). They also showed that phenobarbital and 3-methylcholanthrene pretreatment enhanced metabolism in both species. Unfortunately, Lasker and coworkers (1982) failed to determine if enhanced metabolism in the hen resulted in reduced sensitivity to OPIDN.

Another group (Soliman et al., 1982) examined the comparative delayed neurotoxicity of TOCP and O-ethyl O-(4-cyanophenyl) phenyl phosphonothioate (cyanofenphos) in 2 species with wide differences in sensitivity to OPIDN (hen and mouse). None of the mice treated with multiple doses developed signs of leg weakness or ataxia while all hens did. Soliman and coworkers (1982) also examined the biochemical response of the target for OPIDN in both species. This target, neurotoxic or neuropathy target esterase (NTE), has been shown to be intimately involved in initiation of OPIDN, although subsequent steps after initiation that actually lead to the observed axonopathy are still unclear (refer to

review by M. K. Johnson, 1975). Soliman and coworkers (1982) showed the target NTE was adequately insulted following TOCP or cyanofenphos treatment in hens but not in mice. Their study demonstrated the difference in species sensitivity but did little to offer a potential mechanism for this observed effect.

Our purpose was to examine the potential role of metabolism in the reduced sensitivity of rats to OPIDN. Metabolism was perturbed using piperonyl butoxide and the resultant effect of TOCP was measured. A great deal is known about the role of microsomal metabolism in TOCP delayed neurotoxicity. It has been amply shown that TOCP is metabolized to an active cyclic saligenin phosphate by liver microsomes (Abou-Donia, 1981; Eto, 1974). Formation of this active metabolite is known to occur in species sensitive to OPIDN (e.g. chicken) as well as insensitive species such as the rat (Sharma and Watanabe, 1974; Eto et al., 1962). Furthermore, the active metabolite phosphorylates NTE, the target for OPIDN, whereas the unmetabolized TOCP does not (Sprague and Hendricks, 1982). In light of the role of liver microsomes in producing an active neurotoxic metabolite, findings from this study that piperonyl butoxide, a potent microsomal inhibitor, apparently reduced the resistance of rats to OPIDN are not unexpected. Furthermore, metabolic perturbation may be a useful procedure for developing new models for OPIDN using experimental animals previously thought to be highly resistant to this characteristic type of neurotoxicity.

ACKNOWLEDGEMENT

The excellent technical assistance of Anne Howell, Lance Sandvik, and Monica Hendricks is gratefully acknowledged.

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CHEMISTRY AND METABOLISM OF DELAYED NEUROTOXIC ORGANOPHOSPHORUS ESTERS

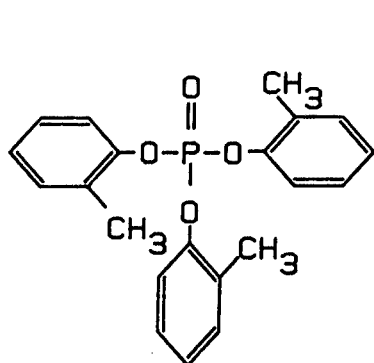
J. Gary Hollingshaus, Ph.D.

Agricultural Research Division
American Cyanamid Company

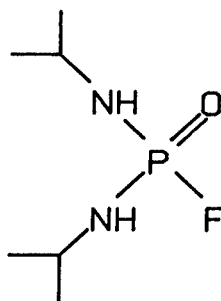
INTRODUCTION

Organophosphorus esters are an important class of chemicals with widespread use as plasticizers, high temperature lubricants, hydraulic fluids, flame retardants, gasoline additives, pesticides, chemical warfare agents, etc. Many of these esters inhibit the enzyme acetylcholinesterase and result in the acutely toxic effects of organophosphorus pesticides and so-called nerve-gases (Fisher and Van Wazer, 1961; Health, 1961). However, some produce a more insidious delayed neuropathy which is manifested as ataxia and paralysis of the extremities of sensitive species 1 to 3 weeks after exposure. This clinical syndrome of humans and domestic animals is currently referred to as organophosphorus ester-induced delayed neurotoxicity (OPIDN) (Abou-Donia, 1981).

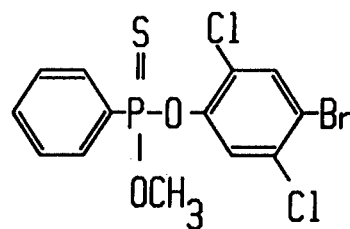
Extensive investigations of the chemical properties of delayed neurotoxic organophosphorus esters resulted from several major incidents of poisoning involving three different compounds (Figure 1). The first was a massive outbreak of neuropathy



TOCP



Mipafox



Leptophos

Figure 1. Chemical structures of TOCP, mipafox, and leptophos.

affecting thousands of people in the mid-western and south-western United States in the late 1920s. A simple triaryl phosphate, tri-2-cresyl phosphate (TOCP), present as an impurity in an alcoholic extract of Jamaican ginger was found to be the causative agent (Smith et al., 1930). Numerous other incidents of poisoning have since been reported from the consumption of TOCP contaminated cooking oils, drinking water, herb extracts, and exposure in the work environment (Davies, 1963). OPIDN was considered to be a unique form of human neuropathy resulting from exposure to TOCP until two research workers involved in manufacturing a new organophosphorus insecticide Mipafox (N,N'-diisopropyl phosphorodiamidofluoridate) were stricken with delayed neurotoxicity while recovering from acute poisoning in the early 1950s (Bidstrup et al., 1953). Most recently, the widespread use of the organophosphorus insecticide Leptophos, (O-(4-bromo-2,5-dichlorophenyl) O-methyl phenylphosphonothioate) in Egypt resulted in the death of some 1300 water buffalo afflicted with OPIDN (Abou-Donia et al., 1974). A subsequent investigation of unusual central nervous system disorders among employees of a Leptophos manufacturing plant found many workers with symptoms compatible with organophosphorus insecticide intoxication (EPA Advisory Committee, 1976; EPA, 1977; Curtis, 1978; Waters and Gerstner, 1979).

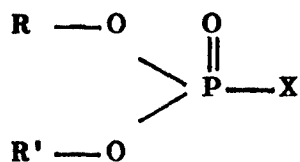
Each of these incidents of poisoning resulted in considerable efforts directed towards an understanding of the molecular characteristics of such delayed neurotoxins and the ability to predict the neurotoxic potential of organophosphorus esters in general. Although some progress has been made in this area, predictions of neurotoxic potential have been limited to a series of closely related compounds. In this paper, a general review of structure/activity relationships, metabolism, and stereochemistry of delayed neurotoxic organophosphorus esters will be presented.

BIOCHEMICAL LESION

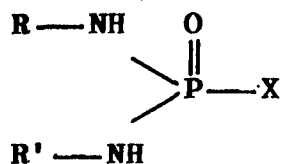
The biochemical lesion responsible for the clinical symptoms of OPIDN is believed to be the phosphorylation of a specific protein receptor in the nerve axon often referred to as "neurotoxic esterase" or "neurotoxic target enzyme" (Johnson, 1969, 1970). In an examination of a variety of compounds for NTE inhibitory activity and delayed neurotoxicity, it became apparent that several non-neurotoxic compounds were also inhibitors of NTE. Further investigation found the non-neurotoxic inhibitors of NTE were not only non-neurotoxic but they actually protected animals subsequently challenged with known delayed neurotoxins. These findings led to a classification of inhibitors of NTE based upon their neurotoxicity. As shown in Figure 2, the neurotoxic inhibitors were phosphates, phosphoramidates, and phosphonates while the non-neurotoxic inhibitors were sulfonates, phosphinates, and carbamates (Johnson, 1974).

The essential difference between these two groups is the ability of the neurotoxic compounds to undergo a second time-dependent reaction called "aging", subsequent to phosphorylation of the

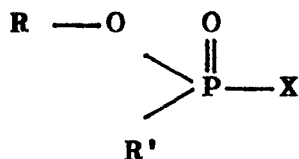
GROUP A



Phosphate

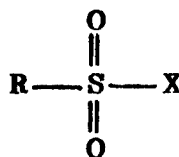


Phosphoramidate

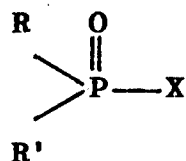


Phosphonate

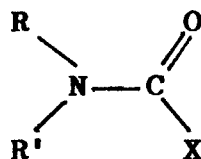
GROUP B



Sulphonate



Phosphinate



Carbamate

Figure 2. Neurotoxic and non-neurotoxic inhibitors of NTE.

A) Neurotoxic

B) Non-neurotoxic

From Johnston, 1975a.

enzyme active site. Aging involves the loss of a R group via dealkylation from the covalently bound phosphorus moiety which results in a negatively charged phosphoryl group. Nucleophilic displacement of the phosphate normally involved in regeneration of the active enzyme is thus prevented and the inhibited enzyme is said to have "aged". Both phosphorylation of NTE and subsequent aging are now thought to be an essential part of delayed neurotoxicity (Johnson, 1974). A recent study further suggests that specific transfer of the R group to a neighboring site via direct alkylation may also be an integral part of the biochemical lesion (Clothier and Johnson, 1979; Johnson, 1982). A more detailed discussion of biochemical aspects of delayed neurotoxicity will be presented later in this conference (Schwab, 1983).

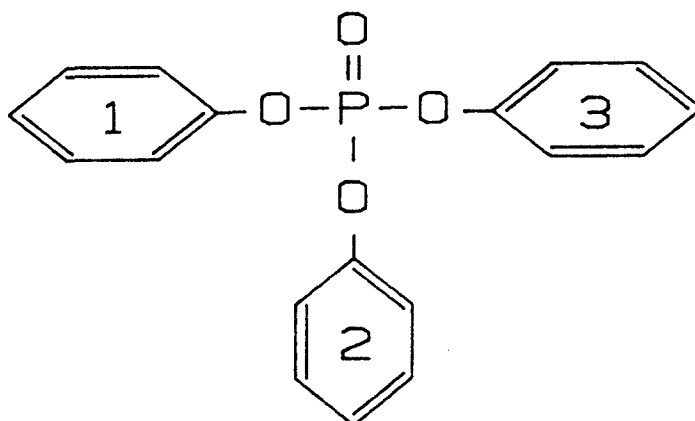
STRUCTURE/ACTIVITY RELATIONSHIPS

Triaryl Phosphates. The rapidly expanding use of triaryl phosphates as plasticizers in the 1940s and 1950s prompted several studies of the acute and delayed neurotoxic properties of such compounds (Hine et al., 1956; Henschler, 1958; Bondy et al., 1960; Aldridge and Barnes, 1961). As shown in Table 1, at least 70 different analogs were examined of which 28 were found to cause delayed neurotoxicity. Simple alkyl substituents in the ortho position of the aromatic rings generally resulted in neurotoxic activity while activity in the para position was limited to ethyl or acetyl substituents. All of the meta substituted analogs were inactive with the exception of one report in which tri-3-methylphenyl phosphate was reported to be neurotoxic at very high doses (Hunter et al., 1944). However, this compound was reported to be non-neurotoxic in three other studies (Smith et al., 1932; Bondy et al., 1960; Johnson, 1975a), and the possibility that an impurity in the test compound may have caused the neurotoxicity has been suggested (Johnson, 1975a).

An explanation for the neurotoxic activity of these compounds was provided when an elegant series of studies showed that TOCP and tri-4-ethylphenyl phosphate are activated in vivo by oxidation of an α -carbon, as shown in Figure 3. In the case of TOCP, hydroxylation of an α -carbon followed by nucleophilic attack on the phosphorus atom results in the displacement of one phenyl ring and the formation of a phenyl saligenin phosphate (Eto et al., 1962). This metabolite was shown to be a potent delayed neurotoxin in vivo (Baron et al., 1962) and in vitro (Johnson, 1975b), and is believed to be the actual toxicant of TOCP. A similar hydroxylation of an α -carbon was observed in the metabolism of tri-4-ethylphenyl phosphate followed by dehydrogenation to form an electron-withdrawing oxo group (Hosli and Henschler, 1970; Eto et al., 1971). This metabolite was also found to be highly neurotoxic. It has been proposed that each of the active compounds listed in Table 1 could be similarly metabolized to form cyclic saligenin phosphates or 4- α -oxo substituents (Johnson, 1975a). The simple triaryl phosphates are rather poor inhibitors of esterases in general while the saligenin phosphates and 4- α -oxo substituted derivatives are significantly better inhibitors. As OPIDN is believed to be the result of phosphorylation of NTE, metabolic activation of many of these compounds is thought to be an essential part of their toxic action.

Although no really comprehensive investigation of the relationship between chemical structure and neurotoxic activity of triaryl phosphates has been conducted, some generalizations have been noted (Davies, 1962; Johnson, 1975a; Johnson et al., 1977; Abou-Donia, 1981).

**TABLE 1. TRIARYL PHOSPHATE ESTERS
TESTED FOR DELAYED NEUROTOXICITY IN CHICKENS**



Substituents			Dose (mg/kg)	Route of Admin.	Delayed neuro- toxicity
1	2	3			
H	H	H	1000 (60) ^a	p.o.	-
2-CH ₃	H	H	50	p.o.	+
2-CH ₃	2-CH ₃	2-CH ₃	25 (1.5)	p.o.	+
2-CH ₃	2-CH ₃	3-CH ₃	250	p.o.	+
2-CH ₃	2-CH ₃	4-CH ₃	25	p.o.	+
2-CH ₃	3-CH ₃	3-CH ₃	50	p.o.	+
2-CH ₃	3-CH ₃	4-CH ₃	50	p.o.	+
2-CH ₃	4-CH ₃	4-CH ₃	50	p.o.	+
2-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	1000	p.o.	+
2,2-di-CH ₃	2,2-di-CH ₃	2,2-di-CH ₃	(12)	p.o.	-
2,3-di-CH ₃	2,4-di-CH ₃	2,3-di-CH ₃	40 x 1000	p.o.	+
2,4-di-CH ₃	2,4-di-CH ₃	2,5-di-CH ₃	8 x 2500	p.o.	+
2,4-di-CH ₃	2,5-di-CH ₃	2,5-di-CH ₃	18 x 2500	p.o.	-
2,6-di-CH ₃	2,5-di-CH ₃	2,6-di-CH ₃	18 x 2500	p.o.	-
2,3-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	50 x 900	p.o.	-
2,4-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	28 x 900	p.o.	-
2,5-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	50 x 900	p.o.	-
2,6-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	30 x 900	p.o.	+
2,4-di-CH ₃	2,4-di-CH ₃	3,5-di-CH ₃	13 x 900	p.o.	+
2,6-di-CH ₃	2,6-di-CH ₃	3,5-di-CH ₃	50 x 900	p.o.	+
2-CH ₃ -4-C ₂ H ₅	2-CH ₃ -4-C ₂ H ₅	2-CH ₃ -4-C ₂ H ₅	2 x 700	p.o.	-
2-C ₂ H ₅	2-C ₂ H ₅	2-C ₂ H ₅	4 x 1200	p.o.	+
2-C ₂ H ₅	2-C ₂ H ₅	4-CH ₃	1000	p.o.	+
2-C ₂ H ₅	4-CH ₃	4-CH ₃	50	p.o.	+
2-C ₂ H ₅	3-C ₂ H ₅	3-C ₂ H ₅	50	p.o.	+
2-C ₂ H ₅	3,5-di-CH ₃	3,5-di-CH ₃	500	p.o.	+
2-n-C ₃ H ₇	4-C ₂ H ₅	4-C ₂ H ₅	100	p.o.	+
2-n-C ₃ H ₇	2-n-C ₃ H ₇	4-CH ₃	4 x 500	p.o.	+
2-n-C ₃ H ₇	4-C ₂ H ₅	4-C ₂ H ₅	100	p.o.	+
2-n-C ₃ H ₇	2-n-C ₃ H ₇	2-n-C ₃ H ₇	1000	p.o.	-
2-n-C ₃ H ₇	3,5-di-CH ₃	3,5-di-CH ₃	1000	p.o.	-

**TABLE 1. (Continued) TRIARYL PHOSPHATE ESTERS
TESTED FOR DELAYED NEUROTOXICITY IN CHICKENS**

Substituents			Dose (mg/kg)	Route of Admin.	Delayed neuro- toxicity
1	2	3			
2-iso-C ₃ H ₇	H	H	10 (12)	p.o.	+
2-iso-C ₃ H ₇	2-iso-C ₃ H ₇	H	600	p.o.	-
2-iso-C ₃ H ₇	2-iso-C ₃ H ₇	2-iso-C ₃ H ₇	1000 (12)	p.o.	-
2-sec-C ₄ H ₉	H	H	1200	p.o.	-
2-tert-C ₄ H ₉	H	H	1200	p.o.	-
2-OCH ₃	2-OCH ₃	2-OCH ₃	3000	p.o.	-
2-C ₆ H ₅	H	H	(120)	p.o.	-
2-C ₆ H ₅	2-C ₆ H ₅	2-C ₆ H ₅	1000	p.o.	-
2-Cl	H	H	1000	p.o.	-
2-Cl	2-Cl	H	1000	p.o.	-
2-Cl	2-Cl	2-Cl	1000	p.o.	-
3-CH ₃	3-CH ₃	3-CH ₃	1200 (25x250)	p.o.	+
3-CH ₃	3-CH ₃	4-CH ₃	2500	p.o.	-
3-CH ₃	4-CH ₃	4-CH ₃	2500	p.o.	-
3,5-di-CH ₃	3,5-di-CH ₃	4-C ₂ H ₅	1000	p.o.	-
3,4-di-CH ₃	3,4-di-CH ₃	3,4-di-CH ₃	18 x 2500	p.o.	-
3,5-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	18 x 2500	p.o.	-
3,4-di-CH ₃	3,5-di-CH ₃	3,5-di-CH ₃	50 x 900	p.o.	-
3-C ₂ H ₅	H	H	1200	p.o.	-
3-C ₂ H ₅	3-C ₂ H ₅	3-C ₂ H ₅	1200	p.o.	-
3-C ₂ H ₅	3-C ₂ H ₅	4-C ₂ H ₅	1200	p.o.	-
3-iso-C ₃ H ₇	3-iso-C ₃ H ₇	3-iso-C ₃ H ₇	1000	p.o.	-
4-CH ₃	3-CH ₃	4-CH ₃	1200	p.o.	-
4-CH ₃	4-CH ₃	4-C ₂ H ₅	2500	p.o.	-
4-CH ₃	4-C ₂ H ₅	4-C ₂ H ₅	1200	p.o.	-
4-C ₂ H ₅	4-C ₂ H ₅	4-CH ₃ -CH(OH)	50	i.m.	+
			70	s.c.	-
			50	i.v.	+
4-C ₂ H ₅	4-C ₂ H ₅	4-CH ₃ -CO ₂	100	p.o.	+
4-C ₂ H ₅	4-CH ₃ -CO ₂	4-CH ₃ -CO ₂	25	i.m.	+
4-CH ₃ -CO ₂	4-CH ₃ -CO ₂	4-CH ₃ -CO ₂	100	p.o.	+
4-CH ₃ -CO ₂	H	H	1000	p.o.	-
4-iso-C ₃ H ₇	H	H	1000 (120)	p.o.	-
4-iso-C ₃ H ₇	4-iso-C ₃ H ₇	H	1000	p.o.	-
4-iso-C ₃ H ₇	4-iso-C ₃ H ₇	4-iso-C ₃ H ₇	1000	p.o.	-
4-sec-C ₄ H ₉	H	H	1200	p.o.	-
4-tert-C ₄ H ₉	H	H	1000 (120)	p.o.	+(-)
4-tert-C ₄ H ₉	4-tert-C ₄ H ₉	H	1000	p.o.	-
4-tert-C ₄ H ₉	4-tert-C ₄ H ₉	4-tert-C ₄ H ₉	450	p.o.	-
4-n-C ₆ H ₁₃	H	H	(120)	p.o.	-
4-nonyl acid	H	H	(120)	p.o.	-

^a Cumulative dose

From Abou-Donia, 1981.

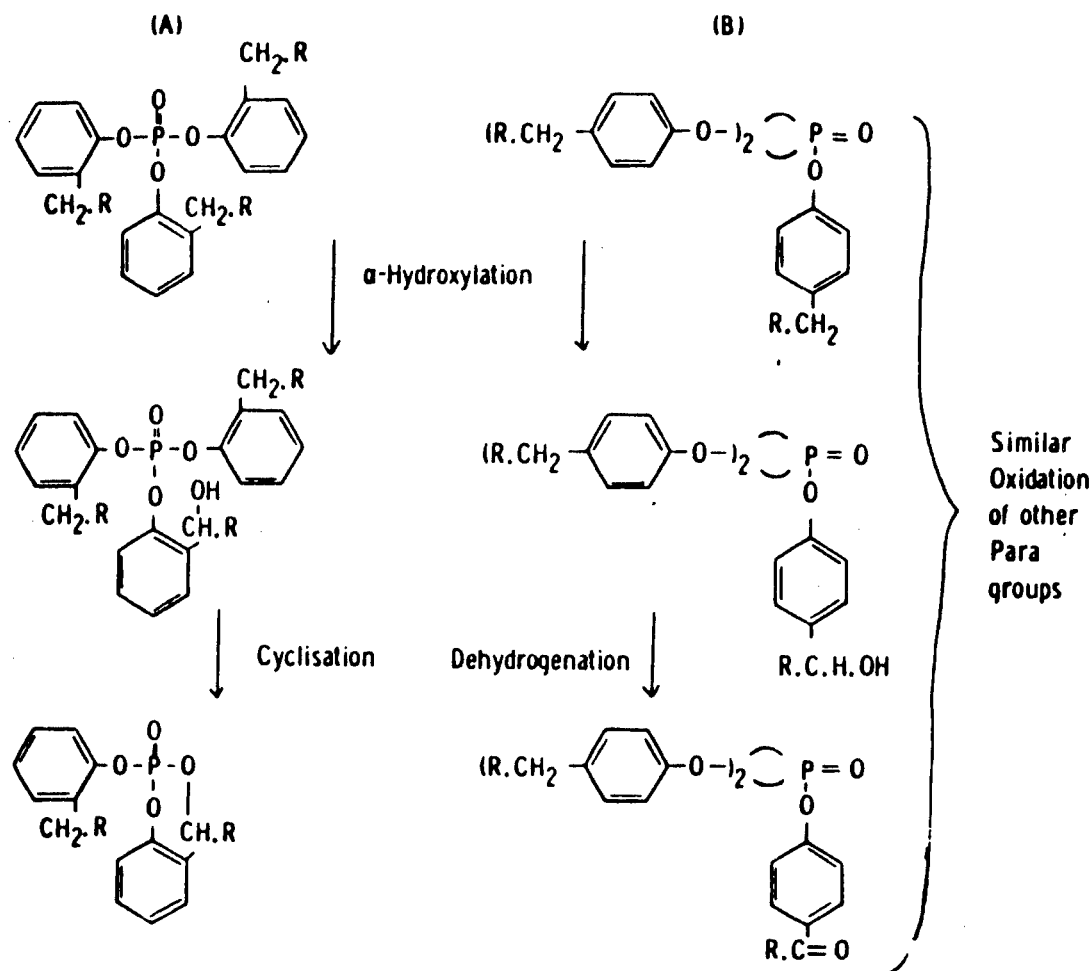


Figure 3. Route of activation of tri-aryl phosphates in vivo: (A) Ortho-substituted; (B) Para-substituted. From Johnson, 1975a.

Esters Having One or More Ortho-Substituted Phenyl Groups

If the ortho-alkyl group has at least one hydrogen on the α -carbon, cyclic derivatives can be formed. Such derivatives are often highly neurotoxic.

If the ortho-substituted ring contains additional substituents, neurotoxicity is markedly reduced. Multiple substituents in other rings does not substantially reduce neurotoxicity.

Isomers having only one ortho-substituent are more neurotoxic than the symmetrical tri-ortho-esters.

Neurotoxic activity declines as the size of the ortho-substituent becomes larger.

Esters Having No Ortho-Substituents

Unsubstituted triphenyl phosphate is not neurotoxic.

Para-substituents require two hydrogen atoms on the -carbon atom to produce an inhibitory -oxo metabolite.

Substituents at the meta position may be metabolized but do not yield neurotoxic products.

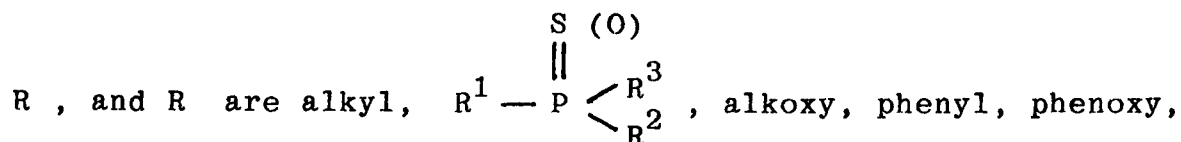
Symmetrical tri-para-substituted esters are more active than mixed para and meta substituted esters.

Methyl groups in the meta or para position reduce the neurotoxicity.

It seems apparent that steric as well as chemical constraints are an important factor in formation of active metabolites from tri-arylphenyl phosphates. Oxidation of the -carbon of both ortho- and para-substituted isomers, which is evidently critical to the formation of the active metabolites, is sterically hindered with increasing substituent size and number. It is also reasonable to assume that the active site has a three-dimensional configuration into which such neurotoxins must fit. Molecules with complementary structures would have access to the active site while other molecules would be excluded or have a less than optimal fit (Abou-Donia, 1981).

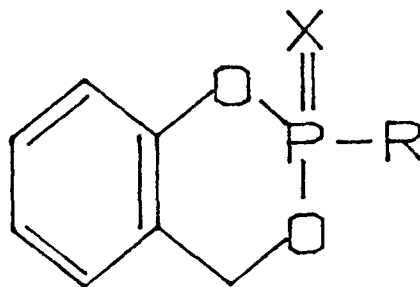
Even though many saligenin phosphates are delayed neurotoxins (Table 2), some which have very favorable insecticidal activity are apparently not or at least are not highly neurotoxic. Salithion (O-methyl saligenin phosphorothioate) has been used for control of a number of pests of rice, fruit, and cotton for many years and was reported to be non-neurotoxic in two early studies (Casida et al., 1963; Eto, 1972). Recently, however, salithion has been found to be neurotoxic in hens given multiple high doses (El-Sebae et al., 1981). Numerous other derivatives of saligenin phosphates have also been examined for insecticidal and neurotoxic activity (Eto et al., 1981; Tawatata et al., 1982), but as yet prediction of delayed neurotoxic potential is not totally reliable.

Alkyl and Aryl Phosphates and Phosphonates. Organophosphorus esters of the general structural formula (I), where R ,



fluoro, and alkylamino substituents, have found widespread use as pesticides (Metcalf, 1959). It has been estimated that more than 500,000 such compounds have been synthesized, of which some 200

**TABLE 2. Saligenin Cyclic Phosphorus Esters
Tested for Delayed Neurotoxicity in Chickens**



<u>R</u>	<u>X</u>	<u>Dose (mg/kg)</u>	<u>Route of Administration</u>	<u>Delayed Neurotoxicity</u>
CH ₃ O	O	12.0	i.p.	-
CH ₃ O	S	80.0	i.p.	+/-
C ₆ H ₅ O	O	2.0	i.p.	+
2-CH ₃ -C ₆ H ₄ O	O	5.0	i.p.	+
3-CH ₃ -C ₆ H ₄ O	O	2.0	i.p.	+
4-CH ₃ -C ₆ H ₄ O	O	0.5	i.p.	+
3,5-di-CH ₃ -C ₆ H ₃ O	O	8.0	i.p.	+
2-Cl-C ₆ H ₄ O	O	25.0	i.p.	+
C ₂ H ₅	O	2.0	i.p.	-
ClCH ₂	O	25.0	i.p.	-
C ₆ H ₅	O	200.0	i.p.	+
C ₆ H ₅	S	100.0	i.p.	+
(CH ₃) ₂ N	O	10.0	i.p.	-

From Abou-Donia, 1981.

have been used commercially (Spencer, 1973). Since the discovery of delayed neurotoxicity of Mipafos and Leptophos, an evaluation of the neurotoxic potential of new candidate pesticides is required by the governments of many countries throughout the world (WHO, 1977; EPA, 1978). Unfortunately, such evaluations are generally limited to compounds with immediate commercial potential while the vast majority of synthesized OP's have not been evaluated. Quantitative structure/activity relationships are therefore limited to a relatively few series of organophosphorus esters. Several excellent reviews of reported studies have been published (Davis, 1963; Johnson, 1975ab; Abou-Donia, 1981; Metcalf, 1982). A few of the more extensive studies have been selected for review in this paper.

Phosphoro- and Phosphonofluoridates. Shortly after Mipafos was found to be a delayed neurotoxin (Bidstrup et al., 1953), DFP (O,O-di-isopropyl phosphorofluoridate) was shown to cause OPIDN in chickens (Barnes and Denz, 1953). Because of the structural similarity of these compounds to the nerve gas Sarin (O-isopro-

pylmethylphosphonofluoridate), Davies et al. 1960 examined a series of 36 related OP esters for OPIDN in the hen. Each of the phosphorofluoridates and phosphonofluoridates tested was delayed neurotoxic. A comparison of the delayed neurotoxic potential of the phosphorofluoridates with relative size of the alkyl substituents (Figure 4) showed a 100-fold increase in activity as the size of the alkyl group was increased from methoxy to n-propoxy and iso-propoxy. Thereafter a decrease in activity was found as the substituent size increased (Davis et al., 1960; Mager, 1981).

NEUROTOXIC PHOSPHOROFUORIDATES

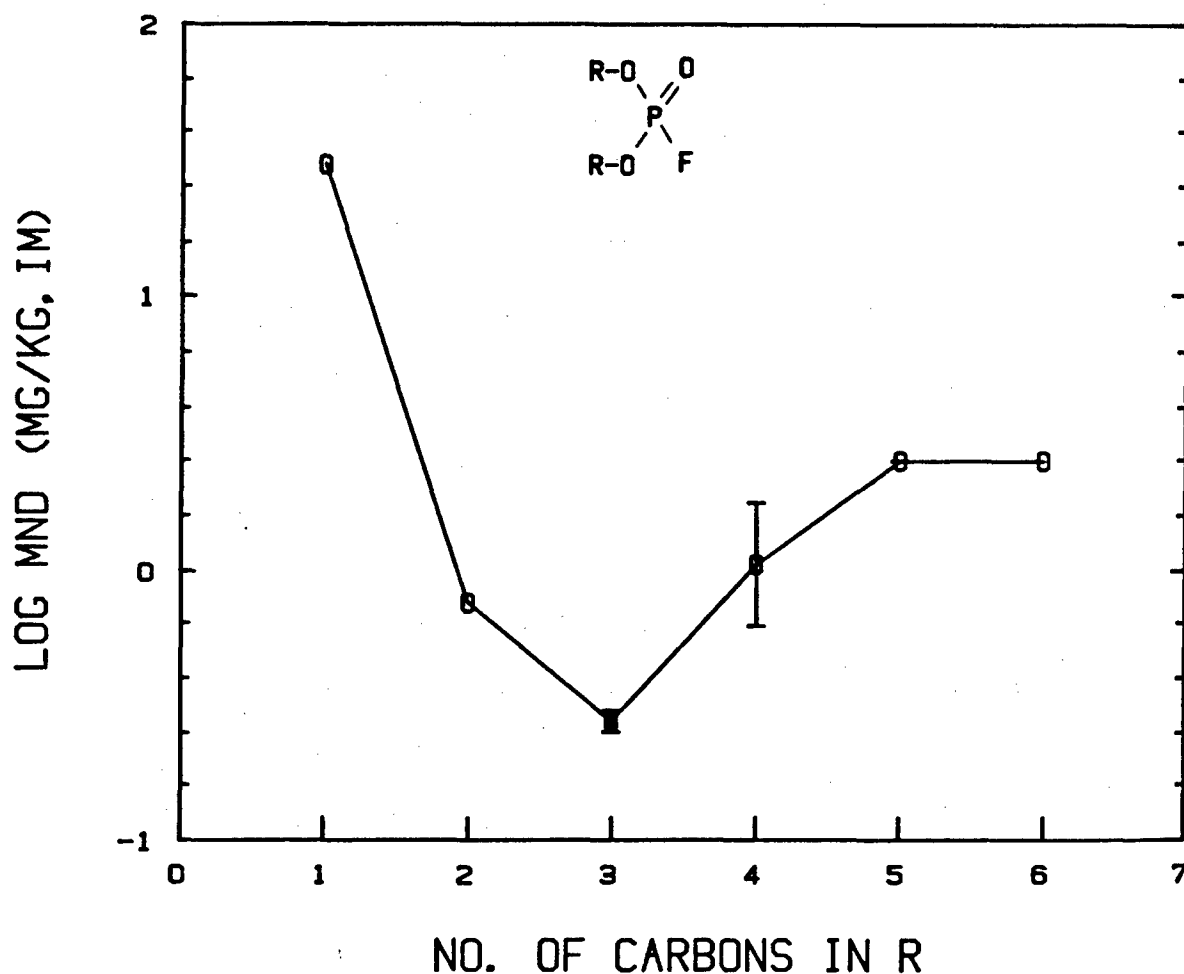


Figure 4. Correlation of substituent size with neurotoxicity for O-alkyl phosphorofluoridates.

A similar correlation was found with a series of N,N'-alkyl phosphorodiamidofluoridates (Figure 5) (Davies et al., 1966). However, the most active compound in this series was the butylamino analog. As all of the neurotoxic compounds tested contained fluorine while the corresponding chloridates were inactive, it was suggested that the fluorine atom must play a direct role in the development of the biochemical lesion, possibly by release of the fluorine at a specific site in the nervous system. Subsequent studies have shown this hypothesis to be incorrect as OPIDN can be produced by similar phosphates and phosphonates with a variety of substituents in place of fluorine.

NEUROTOXIC PHOSPHORODIAMIDOFLUORIDATES

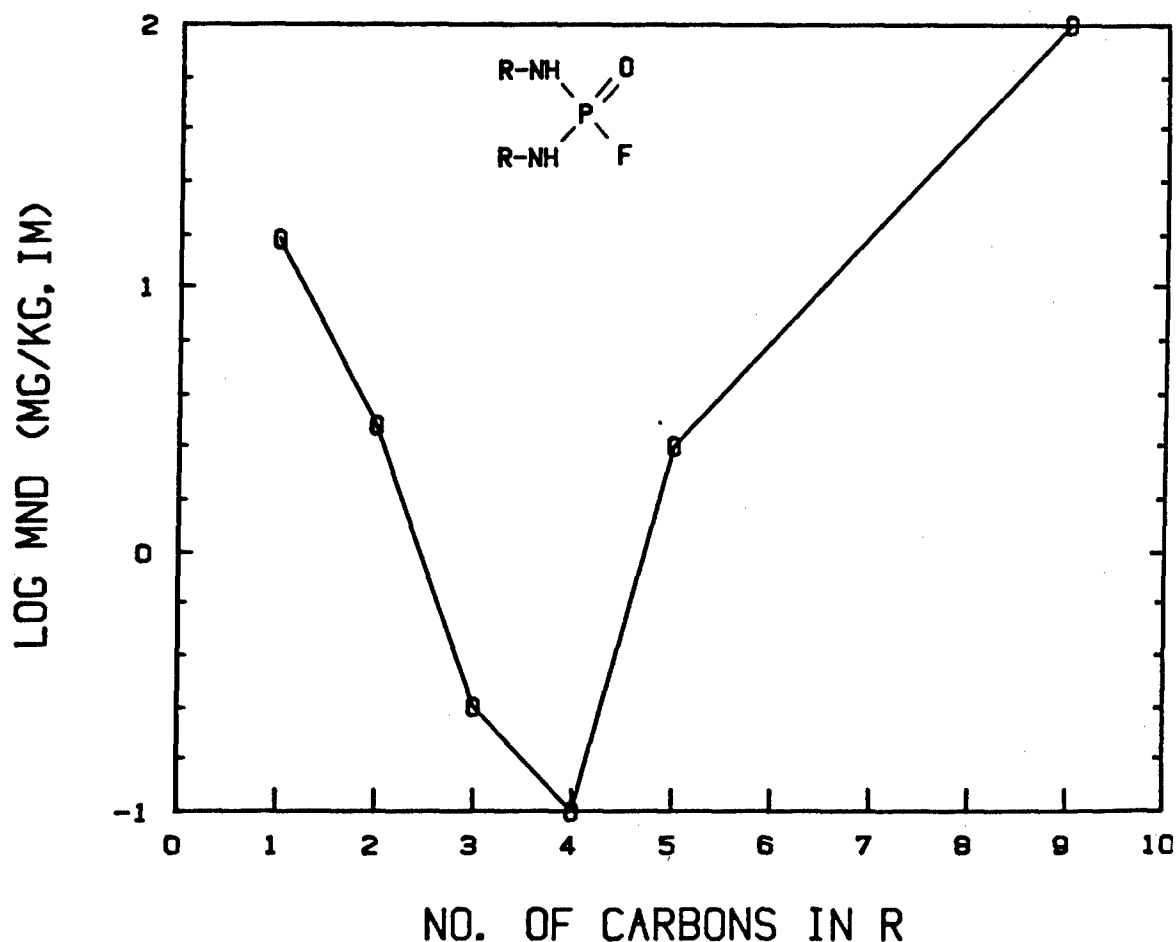


Figure 5. Correlation of substituent size with neurotoxicity for N,N'-dialkyl phosphorodiamidofluoridates.

Dichlorovinyl Phosphates. Dichlorvos (O,O-dimethyl O-(2,2-dichlorovinyl) phosphate and trichlorfon (O,O-dimethyl 1-hydroxy-2,2,2-trichloroethylphosphonate), which undergoes a molecular rearrangement to form dichlorvos, are two widely used pesticides. Conflicting reports of the delayed neurotoxic potential of these compounds have caused some concern about their use (Johnson, 1975a; Hierons and Johnson, 1978; Olajos et al., 1979). In two more recent studies both dichlorvos and trichlorfon were clearly shown to cause OPIDN in hens but the minimum neurotoxic doses were 2-10 times the unprotected LD₅₀. Danger of neurotoxicity from sub-chronic exposure to dichlorvos was considered to be negligible as such exposure would result in severe anticholinesterase effects long before a threshold neurotoxic dose would be reached (Johnson, 1981; Caroldi and Lotti, 1981).

An investigation of the neurotoxic potential of other alkyl dichlorovinyl phosphates found neurotoxicity increased dramatically as the size of the alkyl substituents increased (Johnson, 1975a,b). A comparison of neurotoxic dose and the NTE I₅₀ with substituent size is shown in Figure 6. The maximum in vivo

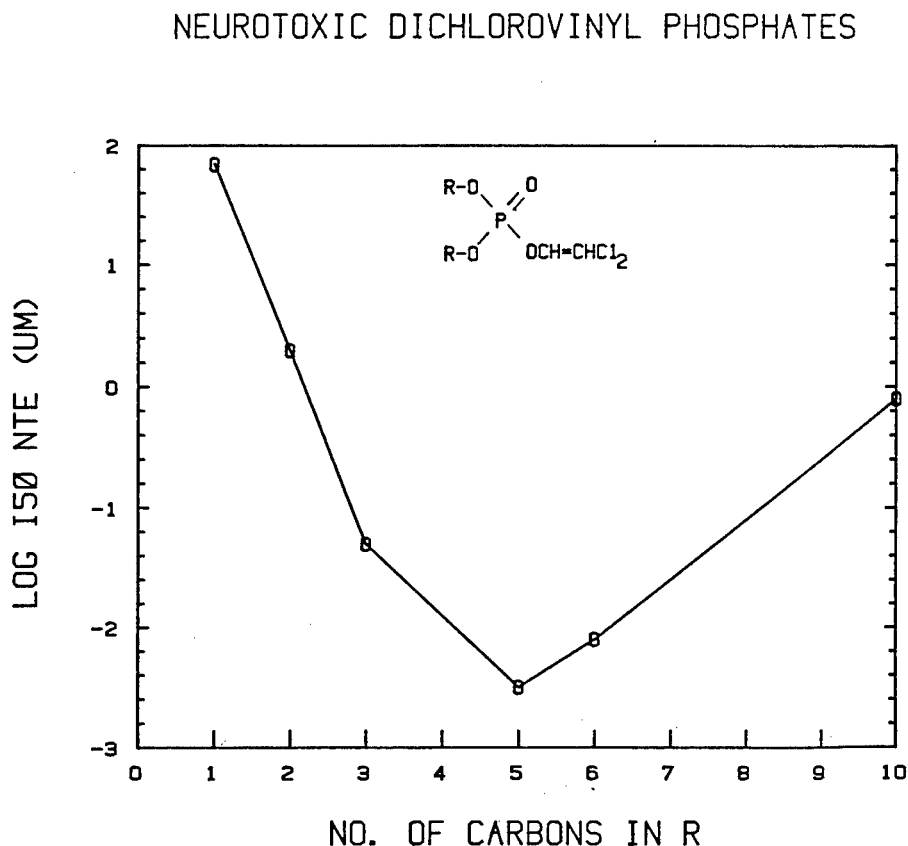


Figure 6. Correlation of substituent size with neurotoxicity of O-alkyl dichlorovinyl phosphates.

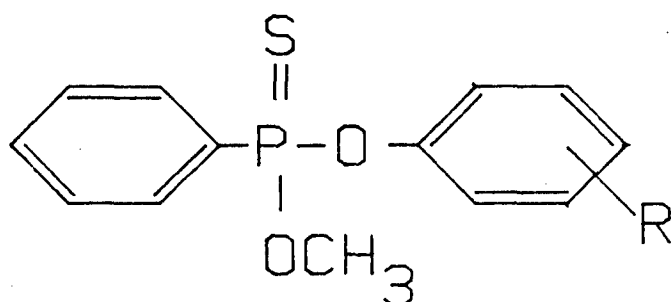
activity was found with the n-propyl and n-pentyl analogs which were 50 times more active than dichlorvos. A similar relationship was observed in vitro where the NTE I₅₀ was 3×10^{-9} M for the n-pentyl analog. Although it is unlikely the OPIDN would result from exposure to dichlorvos or trichlorfon, other alkyl dichlorovinyl phosphates are among the most potent delayed neurotoxins yet described.

The correlation between neurotoxic potential and the size of the alkyl groups of the phosphoro- and phosphonofluoridates, phosphorodiamidofluoridates and dichlorovinyl phosphates are remarkably similar. The most neurotoxic compounds are generally the n-propyl to n-pentyl analogs. If Johnson's hypothesis that the biochemical lesion involves phosphorylation followed by rapid aging of NTE is correct, then a similar correlation should exist between substituent size and rate of aging of NTE. A report of the approximate $t^{1/2}$ values for aging of NTE by various organophosphorus esters does show such a trend but unfortunately does not sufficiently discriminate $t^{1/2}$ values to confirm such a correlation (Clothier and Johnson, 1980). Although the exact mechanism of aging of NTE is not known, evidence suggests that it is not directly comparable to aging of acetylcholinesterase (AChE) (Clothier and Johnson, 1979; Johnson, 1982). The mechanism of aging of phosphorylated AChE is believed to be unimolecular acid catalyzed dealkylation with the rate being dependent upon the stability of the carbonium ion produced by the dealkylation (Berends et al., 1959; Michel et al., 1967). In contrast, aging of NTE appears to involve the specific transfer of an alkyl group to another site in the protein by direct alkylation. Because aging of NTE occurred rapidly with aryloxy and linear alkoxy groups attached to phosphorus but slowly with a highly branched alkoxy substituent, these effects seem incompatible with an S_N1 dealkylation mechanism.

Phenyl- and alkylphosphonothioates. This class of organophosphorus esters has been evaluated extensively as possible replacements for the highly persistent organochlorine insecticides. However, they too are highly lipophilic and substantially resistant to environmental degradation, and several of these compounds developed as commercial insecticides have been shown to cause OPIDN. These include EPN (O-ethyl O-4-nitrophenyl phenylphosphonothioate), leptophos (O-(4-bromo-2,5-dichlorophenyl) O-methyl phenylphosphonothioate), cyanofenphos (O-ethyl O-4-cyanophenyl phenylphosphonothioate), trichloronate (O-ethyl O-(2,4,5-trichlorophenyl) ethylphosphonothioate), and S-Seven (O-ethyl O-(2,5-dichlorophenyl) phenylphosphonothioate) (Abou-Donia, 1979a).

Numerous studies of structure/activity relationships of OPIDN with phenylphosphonates and alkylphosphonates have been published (Johnson, 1975a; Hollingshaus et al., 1979; Francis et al., 1980a,b; Francis et al., 1982; Metcalf et al., 1983). As shown in Table 3, all of the O-methyl O-substituted phenyl phenylphosphonothioates tested were found to produce OPIDN in hens. As phosphorylation of AChE and NTE is considered essential

TABLE 3. TOXICOLOGY OF O-METHYL O-SUBSTITUTED PHENYL PHENYLPHOSPHONOTHIONATES



R	$\Sigma\sigma$	House Fly Topical LD ₅₀ ($\mu\text{g/g}$)	Mouse Oral LD ₅₀ ($\mu\text{g/g}$)	Hen Oral MND (mg/kg)
2-Cl	0.50	1050.0	165	300
3-Cl	0.37	375.0	500	275
4-Cl	0.23	6000.0	500	400
2,3-Cl ₂	0.87	38.0	178	300
2,4-Cl ₂	0.73	35.5	386	100
2,5-Cl ₂	0.87	17.6	99	30
2,6-Cl ₂	1.00	290.0	155	50
3,4-Cl ₂	0.60	30.2	500	300
3,5-Cl ₂	0.75	9.9	364	300
2,3,4-Cl ₃	1.10	16.7	57	1000
2,3,5-Cl ₃	1.24	10.2	14	100
2,3,6-Cl ₃	1.37	38.3	20	50
2,4,5-Cl ₃	1.10	10.1	56	100
2,4,6-Cl ₃	1.23	20.5	93	100
3,4,5-Cl ₃	0.98	15.8	90	300
2,3,5,6-Cl ₄	1.74	60.7	--	1000
2,3,4,5,6-Cl ₅	1.97	134.3	--	1500
2,5-Cl ₂ -4-Br	1.10	11.6	71	300
2,5-Cl ₂ -4-I	1.10	12.6	56	1000
4-NO ₂	1.27	3.1	8	50
4-CN	0.89	10.4	101	500
4-CH ₃ S(O ₂)	1.05	52.0	10	100

From Metcalf et al., 1983.

to acute poisoning and OPIDN, respectively, a correlation between the rates of phosphorylation and acute toxicity or OPIDN should exist. The rate of phosphorylation is a function of the reactivity of the phosphate and is determined by the magnitude of the positive charge (electrophilicity) of the P atom. This is greatly influenced by the electron withdrawing or donating character of the substituents attached to it. In the case of substituted O-phenyl substituents the electron-withdrawing nature of the functional groups is defined by Hammett's sigma constant (σ) (Fukuto and Metcalf, 1956).

A comparison of acute toxicity to house flies and mice with OPIDN in hens for a series of O-methyl O-substituted phenyl phenylphosphonothioates is shown in Table 3. A substantial correlation between $\Sigma\sigma^-$ and log LD₅₀ in both house flies and mice was observed and is defined in the following regression equations (Metcalf et al., 1983):

House Flies: $\log \text{LD}_{50} = -1.74 \Sigma\sigma^- + 2.70$; $r = 0.75$, $n = 14$

Mice: $\log \text{LD}_{50} = 2.67 \Sigma\sigma^- + 5.04$; $r = 0.98$, $n = 11$

Marked steric hinderance of reactivity for 2,6-dichlorophenyl substituted compounds had been previously reported and these substituents were therefore omitted from the regression analysis (Tribble and Trayuham, 1969). Similar correlations are common to other substituted phenyl phosphates (Hansch and Deutsch, 1966) indicating the reactivity of the P atom, as influenced by the electron-withdrawing nature of its substituents, is a primary determinate in both enzyme inhibition and acute toxicity.

Correlations between $\Sigma\sigma^-$ and the minimum neurotoxic dose (MND) to hens, however, were much less clear (Metcalf et al., 1983). The regression equation for all of the dichloro- and trichlorophenyl O-methyl phenylphosphonothioates listed in Table 3 was:

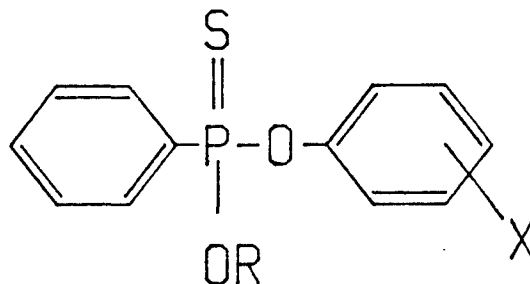
$\log \text{MND} = -0.77 \Sigma\sigma^- + 3.31$; $r = 0.38$, $n = 12$

Some improvement in the correlation was obtained when the di- and tri-substituted analogs were analyzed individually but coefficients were still only -0.60 and -0.69, respectively.

A further complexity was noted when the NTE pI₅₀ was compared with the MND in hens for a group of halogenated-phenyl O-alkyl phenylphosphonates (Table 4). Each of the O-methyl analogs was several times more neurotoxic than the corresponding O-ethyl analogs. A comparison of pI₅₀ values of NTE, however, showed little difference in inhibitory potential between the pairs of analogs (Ohkawa et al., 1980; Francis et al., 1982; Metcalf, 1983; Reinders et al., 1983). Various explanations have been suggested to account for these differences, e.g., absorption, metabolism, aging, etc., but as yet no definitive evidence has been obtained.

It is apparent the acute toxicity of O-halogenated-phenyl O-methyl phenylphosphonothioates and the O-ethyl analogs can be explained almost entirely by factors known to affect the rate of phosphorylation of AChE. In contrast, a much less positive correlation was observed between factors expected to govern the inhibition by phosphorylation of NTE. The unexpectedly high neurotoxic potential of O-(2,6-dichlorophenyl) O-methyl phenylphosphonothioate certainly suggests more subtle events are involved in OPIDN than for inhibition of AChE. Furthermore, the tremendous differences between in vivo and in vitro results for

TABLE 4. CORRELATION BETWEEN pI_{50} FOR NTE AND MND IN HENS FOR HALOGENATED-PHENYL O-ALKYL PHOSPHON-(THIO)ATES



X	R	NTE pI_{50} ^a P = O	MND oral-hen (mg/kg) ^b P = S
2,5-Cl ₂	CH ₃	6.70	30
	C ₂ H ₅	7.23	1500
2,4-Cl ₂	CH ₃	5.95	100
	C ₂ H ₅	6.38	1500
2,4,5-Cl ₃	CH ₃	7.00	100
	C ₂ H ₅	7.05	1500
2,5-Cl ₂ ,4-Br	CH ₃	7.03	250-300
	C ₂ H ₅	7.11	1500

^a Ohkawa et al. 1980

^b Francis et al. 1982.

the O-ethyl and O-methyl phenylphosphonothioates raise numerous questions about the accuracy of predictions of neurotoxic potential.

Nerve Gases. The extreme toxicity of OP nerve agents has been attributed to their rapid inhibition of AChE and aging to form a phosphorylated enzyme refractory to reactivation (Loomis and Salafsky, 1963). The similarity in this lesion to that proposed for OPIDN (Johnson, 1974) suggested the OP nerve agents may also be highly neurotoxic. Recent suspected use of chemical warfare agents against humans has prompted renewed interest in the use and hazards of such compounds. The possibility that delayed neuropathy may develop in survivors of such poisoning was recently examined (Gordon et al., 1983). The in vitro inhibitory potencies of several nerve agents and related OP compounds against AChE and NTE were compared. Although the I_{50} 's against AChE were approximately 0.1-1.0 nM for the most common OP nerve agents, Sarin (O-isopropyl methylphosphonofluoridate), Soman (O-pinacolyl methylphosphonofluoridate), and Tabun (O-ethyl N,N-dimethylphosphoramidocyanate), the I_{50} 's against NTE were 2-4 orders of magnitude higher. From in vitro data it was therefore

predicted that nerve agents would cause OPIDN only at doses greatly exceeding the LD₅₀. In hens protected against acute toxicity by pretreatment with atropine, physostigmine, and the oxime P2S (1-methyl,2-hydroxyiminomethylpyridinium methanesulphonate), delayed neuropathy was found at 30-60 times the LD₅₀ for tabun. For the latter two compounds the inhibition of NTE was 55% and 66%, respectively, from which the MND was calculated to be 100-150 times the LD₅₀ for these compounds. From these results it was concluded that with recent advances in therapeutics for OP poisoning consideration must be given to the possibility of the occurrence of neuropathic symptoms in survivors of poisoning in man after exposure to OP nerve gases.

Impurities in Organophosphorous Esters. Many technical products of OP esters contain appreciable amounts of impurities that may represent a significant hazard to humans and animals. Thousands of incidents of OPIDN have been attributed to TOCP present as an impurity or minor product of lubricating and mineral oils (Davies, 1963). Desbromoleptophos, an impurity in technical leptophos and a photolytic product, was found to be 10 times more neurotoxic than leptophos and is thought to be a major toxicant in the paralysis of water buffalo in Egypt (Sanborn et al., 1977).

An exhaustive study of effects of impurities in ethyl leptophos (O-(4-bromo-2,5-dichlorophenyl) O-ethyl phenylphosphonothioate) on OPIDN found the technical product (89%) was approximately twice as active as the purified product (99+) (Table 5)

TABLE 5. DELAYED NEUROTOXIC ACTIVITY OF TECHNICAL VS. PURIFIED ETHYL LEPTOPHOS AS SINGLE ORAL DOSES TO HENS

Compound	Dose (mg/kg)	Mean Days After Treatment When Ataxia Was Observed			
		T1 ^a	T2	T3	T4
Technical (89% pure)	1,500	14	16	19	21
	1,250	14	16	18	21
	1,000	14	20	30	^b
	750	--	23	26	30
Purified (90+% pure)	1,500	24	25	27	29
	1,250	21	23	24	27
	1,000	25	--	--	--
	750	--	--	--	--

^a Based on a scale of 0 to 4 as described by Davies and Holland (1972)

^b Stage of ataxia was not observed

From Hollingshaus et al. (1981).

(Hollingshaus et al., 1981). Of the 11 impurities present in the technical material none of the aryl phenylphosphonate esters caused any sign of neuropathy. Yet, surprisingly, O,O-diethyl phenylphosphonothioate, its oxon analog, and O,O-diethyl 4-chlorophenylphosphonothioate each caused severe paralysis at doses 10-15 times lower than the MND for ethyl leptophos (Tables 6 and 7).

TABLE 6. DELAYED NEUROTOXICITY OF SOME IMPURITIES IN TECHNICAL ETHYL LEPTOPHOS ADMINISTERED ORALLY TO HENS

Compound	No. Hens Treated	Dose (mg/kg) ^a	No. Ataxic ^b
O-(4-Bromo-2,5-dichlorophenyl) O-ethyl phenylphosphonothioate	3	1,250	3
O-(2,5-Dichlorophenyl) O-ethyl phenylphosphonothioate	3	1,000	0
O,O-Bis-(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate	3	1,000	0
O-(4-Bromo-2,5-dichlorophenyl) S-ethyl phenylphosphonothioate	3	200	0
O-(4-Bromo-2,5-dichlorophenyl) O-ethyl 4-chlorophenylphosphonothioate	3	100 ^c	0
O,O-Diethyl phenylphosphonothioate	3	500	3
O,O-Diethyl phenylphosphonate	3	250	3
O,S-Diethyl phenylphosphonothioate	3	500 ^d	0
O,O-Diethyl 4-chlorophenylphosphonothioate	3	200	3

^a Highest dose tested

^b Showing any sign of ataxia

^c Tested both orally and intraperitoneally

^d Required several doses of atropine for survival

From Hollingshaus et al. (1981).

TABLE 7. DELAYED NEUROTOXICITY OF O,O-DIETHYL ESTERS OF PHENYLPHOSPHONOTHIOATE, PHENYLPHOSPHONATE, AND 4-CHLOROPHENYLPHOSPHONOTHIOATE

Compound	Dose (mg/kg)	No. treated	Mean Days After Treatment When Ataxia Was Observed			
			T1	T2	T3	T4
O,O-Diethyl phenylphos- phonothioate	500	3	- ^a	10	11	13
	300	3	--	12	13	15
	200	3	--	11	12	16
	150	3	--	--	--	--
	100	3	--	--	10	14
O,O-Diethyl phenylphos- phonate	250	3	--	--	10	13
O,O-Diethyl 4-chloro- phenylphosphonothioate	200	3	11	12	15	18
	150	1	--	14	16	18
	125	1	--	--	--	--
	100	3	--	--	--	--

^a Stage of ataxia was not observed

From Hollinghaus et al. (1981).

The unexpectedly high neurotoxic activity observed for these simple O,O-diethyl phenylphosphonates is of considerable interest since, in contrast to other neurotoxic organophosphorus esters, these compounds would not be considered potent inhibitors of esterases. It seems evident that the neurotoxic potential of untested organophosphorus esters must be assayed with the technical product as well as highly purified analytical standards in order to properly assess neurotoxic potential.

METABOLISM

Organophosphorus esters are subject to a wide variety of metabolic reactions in living organisms. Major biotransformation reactions are common to compounds with similar structures and are mediated primarily by mixed-function oxidases (mfo's), glutathione S-transferases, and arylesterases. The mixed-function oxidases are a group of membrane-bound enzymes which catalyze an astonishing array of oxidative transformations. The general types of reactions catalyzed by these enzymes have been described as: (1) O-, S- and N-alkyl hydroxylation and dealkylation; (2) aliphatic hydroxylation and epoxidation; (3) aromatic hydroxylation; (4) ester oxidation of OP esters; and (5) nitrogen or thioether oxidation (Nakatsugawa and Morelli, 1976). Such transformations are common to OP esters and may result in either activation or detoxification. The conversion of TOCP to 3-methylphenyl saligenin phosphate and of tri-4-ethylphenyl phosphate to

its oxo-analog (Figure 3) as a result of mfo-mediated O-alkyl hydroxylation were shown to be important activation processes in OPIDN (Eto et al., 1962; Eto et al., 1971). The metabolic conversion of thiophosphoryl (P=S) esters to the corresponding phosphoryl (P=O) esters (Figure 7) via mfo-mediated oxidative desulfuration is also a well known activation reaction occurring in many organisms. Since P=S esters are generally poor inhibitors of AChE and NTE, activation to the P=O esters is considered to be essential for intoxication by thiono-OP esters (Metcalf, 1955).

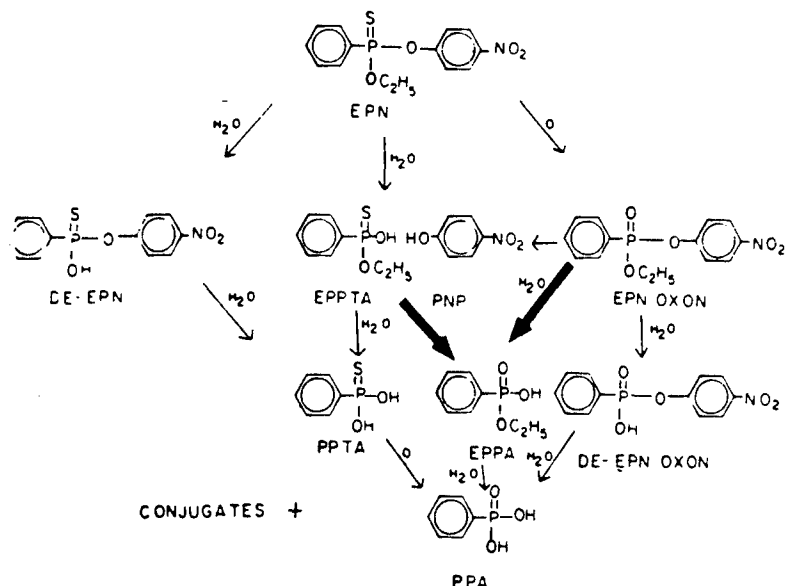


Figure 7. Metabolic transformations commonly observed with organophosphorus esters. From Lasker et al. (1982).

Ester hydrolysis on the other hand is often the major step in the detoxification of OP esters and may be catalyzed by both mfo's and arylesterases. Extensive studies of the metabolic fate of EPN, leptophos, and cyanofenphos found this metabolic pathway to be the primary route in the detoxification of these compounds (Ohkawa et al., 1977a; Abou-Donia, 1979; Lasker et al., 1982). Hydrolysis of both the phosphonothioates and phosphonates was observed. If hydrolysis of the thiono analog occurs prior to oxidative desulfuration to the oxon, formation of the active toxicant is circumvented and intoxication does not occur. A similar detoxification mechanism is O-dealkylation which may be mediated by either mfo's or glutathione S-transferases.

The ultimate toxicity of an OP ester to a given organism will depend upon the sensitivity of a biochemical lesion to insult by the OP and the complex interactions involved in the metabolism of the compound. Detailed comparisons of the metabolic fate of neurotoxic OPs in susceptible and non-susceptible species have shown some variation between species which may be at least partially responsible for the differences in sensitivity (Ohkawa et al., 1977; Abou-Donia, 1979; Ohkawa, 1982; Lasker et al., 1982).

STEREOCHEMISTRY

It is well known that chirality at the phosphorus atom of an organophosphorus ester often has a significant effect on the biological activity of the ester. The difference in toxicity of the enantiomers of chiral organophosphorus poisons has been attributed to differences in their ability to inhibit acetylcholinesterase and other esterases and to differences in their rates of metabolism in animals (Lee et al., 1978).

Recently, the effect of phosphorus chirality on the acute and delayed neurotoxicity of the enantiomers of EPN, cyanofenphos, leptophos, and desbromoleptophos has been described (Ohkawa et al., 1977b; 1980; Nomeir and Dauterman, 1979; Allaharyi et al., 1980; Abou-Donia et al., 1980). As shown in Table 8, the (R)p (+) enantiomers of each of the compounds are more acutely toxic to house flies and mice than the corresponding (S)p (-) enantiomers. In contrast, the (S)p (-) enantiomers are more potent delayed neurotoxins.

TABLE 8. ACUTE AND DELAYED NEUROTOXICITY OF THE CHIRAL ISOMERS OF EPN, CYANOFENPHOS, LEPTOPHOS, AND DESBROMOLEPTOPHOS

Compound	LD ₅₀		Hen MND (i.p.) (mg/kg)
	House Fly (μ g/g)	Mouse (oral) (mg/kg)	
EPN			
Racemic	1	28	53
(R)p (+)	1	32	-89
(S)p (-)	3	31	41
Cyanofenphos			
Racemic	2	34 (i.p.)	
(R)p (+)	1	32 (i.p.)	
(S)p (-)	4	35 (i.p.)	
Leptophos			
Racemic	11	55	150
(R)p (+)	10	51	-100
(S)p (-)	17	64	100
Desbromoleptophos			
Racemic	14	72	10
(R)p (+)	6	64	10
(S)p (-)	22	75	5

Data from: Nomeir and Dauterman, 1979; Ohkawa et al., 1977; Allahyari et al., 1980.

A comparison of the inhibitory activity of the chiral isomers of these same compounds against AChE and NTE in vivo and in vitro shows the same stereochemical relationship exists. As

shown in Figure 8, the (R)p (+) enantiomer of EPN was the more potent inhibitor of AChE in the hen while the (S)p (-) enantiomer was more active against NTE. In vitro, the (R)p (+) enantiomers of the oxons were approximately 4 times more active as inhibitors of AChE than the (S)p (-) enantiomers. Against NTE the inhibitory activity of the enantiomers was just the reverse (Table 9).

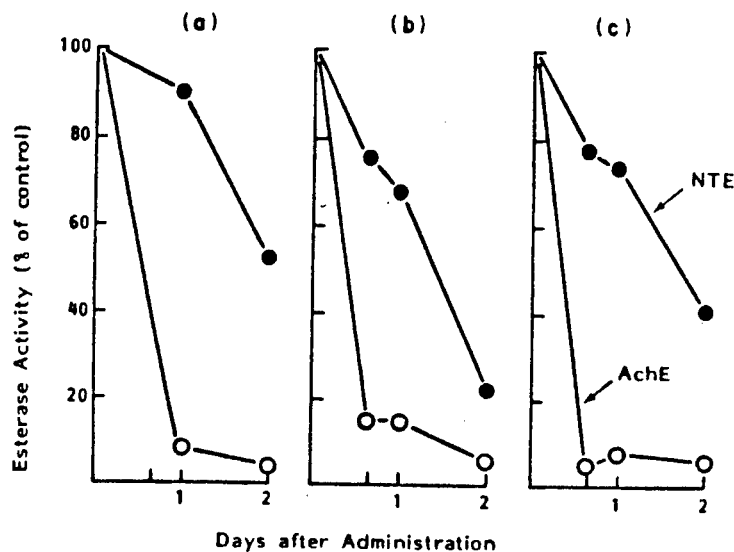


Figure 8. Neurotoxic esterase (NTE) and acetylcholinesterase (AChE) activities in the brain after single subcutaneous administration of 50/mg/kg of (a) (R)p (+)-EPN; (b) (S)p (-)-EPN; and (c) racemic EPN to atropinized hens. From Ohkawa et al. (1980).

Although NTE does exhibit a distinct stereoselectivity among chiral OP inhibitors, it is not totally stereospecific. Furthermore, the greater neurotoxicity of the (S)p (-) enantiomers in vivo and the greater inhibition of NTE by the (S)p (-) oxons in vitro indicate that the metabolic activation of the P=S to the P=O must proceed predominantly with retention of configuration. Similar findings were previously noted with the insecticide fonofos (Lee et al., 1976) and the herbicide S-2571 (Ohkawa et al., 1976).

These findings add further support to the hypothesis that the biochemical lesion responsible for OPIDN is distinctly different from the lesion responsible for acute toxicity. The active site associated with OPIDN is apparently different from the AChE active site, but much more research on the biochemical characteristics of NTE and OPIDN is needed.

**TABLE 9. INHIBITORY ACTIVITY OF THE CHIRAL ISOMERS OF
DELAYED NEUROTOXIC ORGANOPHOSPHORUS COMPOUNDS
AGAINST AChE AND NTE OF HEN BRAIN**

<u>Compound</u>	<u>I₅₀ (uM)</u>		<u>AChE/NTE</u>
	<u>AChE</u>	<u>NTE</u>	
EPN-oxon			
Racemic	0.03	2.14	0.014
(R)p (+)	0.02	2.56	0.008
(S)p (-)	0.08	1.68	0.046
Cyanofenphos-oxon			
Racemic	0.70	4.63	0.151
(R)p (+)	0.36	5.78	0.062
(S)p (-)	1.77	3.68	0.481
Deshromoleptophos-oxon			
Racemic		0.26	
(R)p (+)		0.35	
(S)p (-)		0.21	

Data from: Ohkawa et al., 1980; Fya and Fukuto, 1983.

CONCLUSIONS

In general, correlations between OPIDN in vivo and physical/chemical parameters or inhibition of NTE in vitro have not been totally satisfactory. Some correlations of OPIDN with leaving groups, length of O-alkyl chains, or the nature of the P-C group have been demonstrated, but such correlations only apply to closely related series of compounds where two of the substituents remain constant while the third is varied. Although the biochemical and physiologic functions of NTE are unknown, there are numerous satisfactory correlations between inhibitors of NTE in vitro and OPIDN in vivo. However, significant exceptions have been found which at present make it impossible to predict those structures most likely to produce OPIDN.

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PATHOLOGY OF ORGANOPHOSPHORUS-INDUCED DELAYED NEUROTOXICITY

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INTRODUCTION

Organophosphorus compounds have several effects on the nervous system. One, which is the major focus of this conference, is the symmetrical, distal nerve fiber degeneration (polyneuropathy) produced in a delayed fashion following a single, (or multiple, small) exposure to certain organophosphorus compounds. The term neurotoxic compound is applied here to organophosphates having the ability to elicit such a response in susceptible animals (Davis and Richardson, 1980). The resulting syndrome is termed delayed neuropathy.

Details of the nature and metabolic effects of the neurotoxic organophosphorus compounds, experimental protocols used, species susceptibility, and clinical disease produced in studies of delayed neuropathy are considered elsewhere in these proceedings. This presentation will emphasize the nature of associated nervous system lesions. To properly appreciate this, a brief note of clinical abnormalities is useful. Administration of an appropriate dose of a neurotoxic organophosphorus compound to a susceptible species produces clinical signs in about 1-3 weeks. In hens given tri-ortho-tolyl (or cresyl) phosphate (TOTP), an unsteady, clumsy gait began in about 8-10 days, and in association with ataxia, progressed to weakness and paralysis (Cavanagh, 1954). Wings were affected later, and to a lesser degree. A somewhat similar clinical picture was seen in the cat (Cavanagh, 1964; Bouldin and Cavanagh, 1979a), and other susceptible species (Abou-Donia, 1981; Ahmed and Glees, 1971; Jortner et al., 1983).

NEURONAL CELL BIOLOGY

As will be seen below, the primary target cell in organophosphorus-induced delayed neuropathy is the neuron. A brief overview of some structural and functional features of this cell will help in appreciating the lesions to be described later.

Neurons are a varied group of cells which have been modified for intercellular communication to enable the body to sense internal and external environmental changes, and to respond to them. A prototype neuron can be envisaged by studying a spinal

motor neuron. This cell is made up of a nerve cell body, the nucleus, and perikaryal cytoplasm and its plasma membrane, located in the ventral (anterior) horn of the spinal cord. A series of arborized perikaryal processes form the dendrites. A single, elongated axon arises from the axon hillock and courses through the ventral spinal root and peripheral nerve, to innervate skeletal muscle fibers. The axon acquires multiple segments of myelin shortly after its point of origin.

In addition to activities common to all cells (energy production, maintenance of intracellular environment, etc.), specialized functions are carried out by each region of the neuron (Price and Griffin, 1980). The plasma membranes of the dendrites, cell body, and proximal portion of the axon serve as receptors and transducers of synaptic inputs. The cell body synthesizes proteins and glycoproteins, and the axon, by virtue of several transport systems, is the site of bidirectional movement of such substances. The axolemma of the initial segment of the axon and nodes of Ranvier generate and propagate action potentials. Chemical communication between nerve and skeletal muscle occurs at synaptic terminals and post-synaptic receptors.

Alterations in some of these cell systems are suspected in the development of lesions of organophosphorus-induced delayed neuropathy.

LIGHT MICROSCOPIC STUDIES

Organophosphorus compounds have produced human delayed neuropathy under a variety of conditions (Davis and Richardson, 1980; Casida and Baron, 1976). Because of their wide use in agriculture and manufacturing, considerable potential still exists for development of this neuropathy in man. Although the significance of the human disease is recognized, this presentation will focus on the lesions produced by these neurotoxic compounds in experimental animals. The latter have been both better studied and better related to pathogenetic mechanisms.

Although lesions of delayed neuropathy can be elicited in a number of animal species with organophosphorus compounds, most of the detailed morphologic studies have used the chicken (Gallus gallus domesticus) and cat (Felis domesticus). In these, as in other susceptible species, there is a general similarity in the nature and distribution of lesions, regardless of which neurotoxic organophosphorus compound is used. Two of the latter have been most frequently used in studies to determine the nature and sequence of morphologic change in the nervous system. Of these, TOTP is a compound requiring activation by hepatic microsomal enzyme systems to achieve the neurotoxic state, and diisopropyl fluorophosphate (DFP), is a direct acting neurotoxin. The pathologic effects of a range of other such compounds, including methyl 2,5-dichloro-4-bromophenyl phenylphosphonothionate (leptophos), O-ethyl O-4-cyanophenyl phenylphosphonothioate (cyanofenphos), S,S,S-tributyl phosphorotrithioate (DEF), ethyl

4-nitrophenyl phenylphosphonothionate (EPN), and O,O di-(2-chloroethyl)-O-(3-chloro-4 methyl-7-coumarinyl) phosphate (haloxon), have also been studied (Abou-Donia et al., 1979, 1983; Abou-Donia and Graham, 1979a,b; Preissig and Abou-Donia, 1979; Jortner et al., 1983).

Neurotoxic organophosphorus compounds induce delayed degradative changes in myelinated nerve fibers of the central and peripheral nervous systems. The primary site of injury appears to be the axon, with preferential involvement of the more distal (not terminal) regions of the larger, longer fibers, and with subsequent fiber breakdown below this site (Bouldin and Cavanagh, 1979a,b). Such lesions are best seen in spinal cord, brain stem, and peripheral nerve.

In the spinal cord, the longer tracts are the major site of lesions, and are mainly affected in their more distal regions. Thus, ascending pathways such as the spinocerebellar and gracilis tracts would show most profound degree of involvement in rostral cervical regions, while descending ones such as the cortico-spinal, reticulospinal, and a ventromedial tract (in hens) would be most severely affected in lumbosacral regions (Beresford and Glees, 1963; Cavanagh, 1954; Cavanagh and Patangia, 1965) (Figure 1). Lesions noted in the brain stem and cerebellar white matter are rostral continuations of the affected ascending spinal pathways.

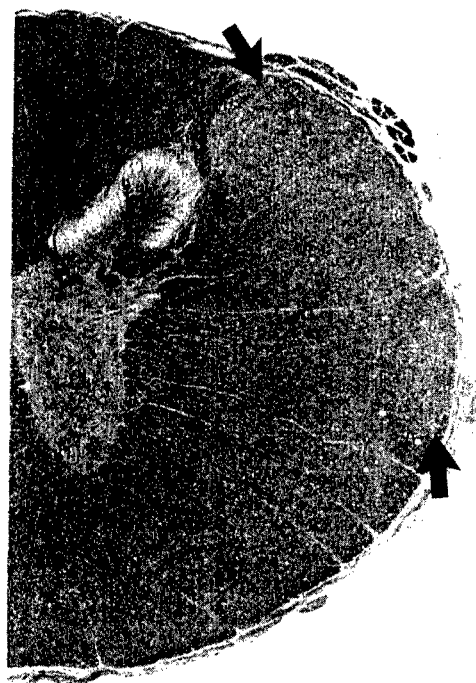


Figure 1. Upper cervical spinal cord from a sheep susceptible to haloxon, one month after 800 mg/kg of that organophosphorus compound was orally administered to the animal. Vacuolated, pale staining regions of white matter are present in the lateral funiculus, in the region of the spinocerebellar tracts (arrows). Luxol fast blue-periodic acid Schiff stain, x15. Reproduced with permission of publishers of Neurotoxicology (from Jortner et al., 1983).

Central nervous system lesions have best been studied in the spinal cord, and at the light microscopic level are manifest by axonal swelling and argyrophilia in the early stages, which proceeds on to fragmentation and degeneration (Beresford and Glees, 1963; Cavanagh, 1954; Cavanagh and Patangia, 1965; Preissig and Abou-Donia, 1978) (Figure 2). Associated closely with the axonal alteration is secondary destruction of the myelin sheath of affected fibers, producing a wallerian-like degeneration (Cavanagh, 1954).

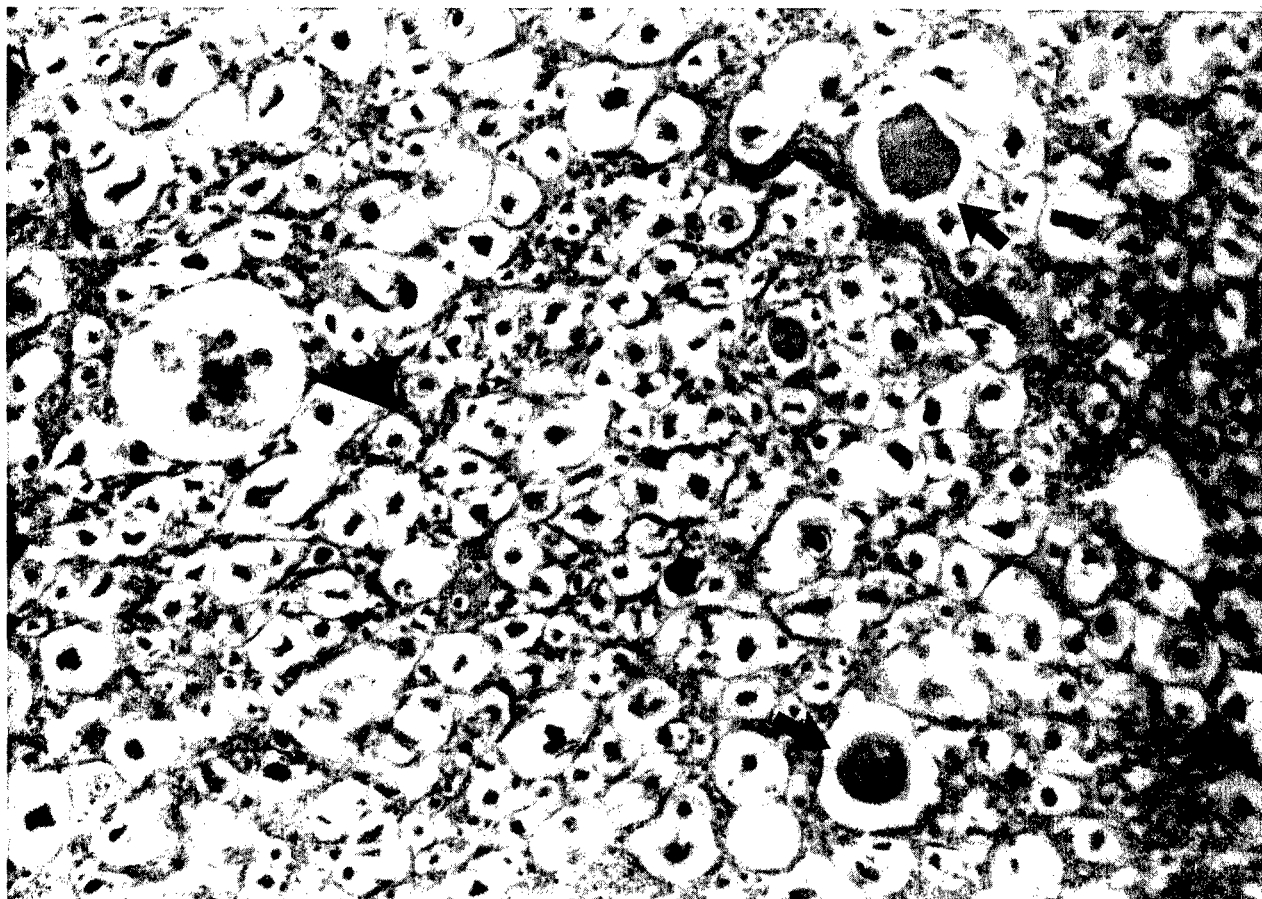


Figure 2. Spinocerebellar tract from an animal similar to that of Figure 1. Swollen axons cut in cross-section (arrows) and a vacuole with nerve fiber debris (arrow-head) are present. Holmes' silver impregnation, x650. Reproduced by permission of publishers of Neurotoxicology (from Jortner et al., 1983).

Several features of these lesions should be noted. Fiber tract lesions generally coincide with the onset of clinical signs (Cavanagh, 1954; Preissig and Abou-Donia, 1978). With clinical progression, additional nerve fibers begin to break down, and study of an affected level of a tract will reveal lesions in

various stages of evolution. As clinical progression ends, spinal tract lesions are mainly in the advanced state (Cavanagh, 1954). No regeneration of spinal cord lesions is seen.

The evolving nerve fiber lesions in affected central nervous system tracts are associated with increased numbers of microglia, macrophages, and reactive astrocytes (Ahmed, 1970; Cavanagh, 1954; Cavanagh and Patangia, 1965; Preissig and Abou-Donia, 1978).

In animals with organophosphorus induced delayed neuropathy, some perikaryal alterations, including changes resembling central chromatolysis, have occasionally been seen in neurons which presumably have degenerating axons (Ahmed and Glees, 1971; Janzik and Glees, 1966; LeVay et al., 1971; Prineas, 1969). These are likely not primary lesions, which appear to be located in more distal regions of the nerve fiber. Cavanagh and Patangia (1965) describe changes in the neuron of the nucleus gracilis interpreted as trans-synaptic degeneration in cats with TOTP poisoning.

Lesions of peripheral nerves in organophosphorus-induced delayed neuropathy are most frequent and best developed in distal regions of longer, larger fibers, resembling those in the spinal cord. Hence, longer nerves to the hindlimbs are more severely affected than the somewhat shorter ones in the forelimb. Distal branches of the sciatic nerve, such as the common peroneal and plantar nerves are frequent sites of injury (Cavanagh, 1964; Prineas, 1969). Degenerative nerve fiber changes are seen even more peripherally, and extend to intramuscular branches and motor or sensory endings (Cavanagh, 1964; Glazer et al., 1978). The recurrent laryngeal nerve in cats is also affected (Bouldin and Cavanagh, 1979a).

Peripheral nerve lesions are seen by light microscopy to begin about the time of clinical onset, with axonal swelling and associated fragmentation of the myelin sheath (Cavanagh, 1954) (Figure 3). This progresses to complete breakdown and phagocytosis of the nerve fiber in association with Schwann cell and macrophage proliferation (Figure 4). This process affects whole lengths of nerve fiber, and resembles wallerian degeneration. Numbers of involved fibers vary with level of nerve examined, time following toxin administration, and effective neurotoxic dose. Cats given varying doses of TOTP developed degenerative changes in 10-50% of plantar motor nerve terminals (Cavanagh, 1964). Another study using the same animal and toxin revealed early (2-7 days) in the course of the disease, some 15% of intramuscular nerve branches had lesions, and at a later stage 25-40% were involved (Prineas, 1969). Mid to distal levels of the recurrent laryngeal nerve of cats had lesions in 1-2% of myelinated nerve fibers, 14 days after exposure to neurotoxic doses of DFP (Bouldin and Cavanagh, 1979a). By 21 days, almost 50% of such fibers were involved, with the majority undergoing wallerian-like degeneration. As in the spinal cord, at any one time, lesions in varying stages of evolution will be evident in affected peripheral nerves during clinical progression.

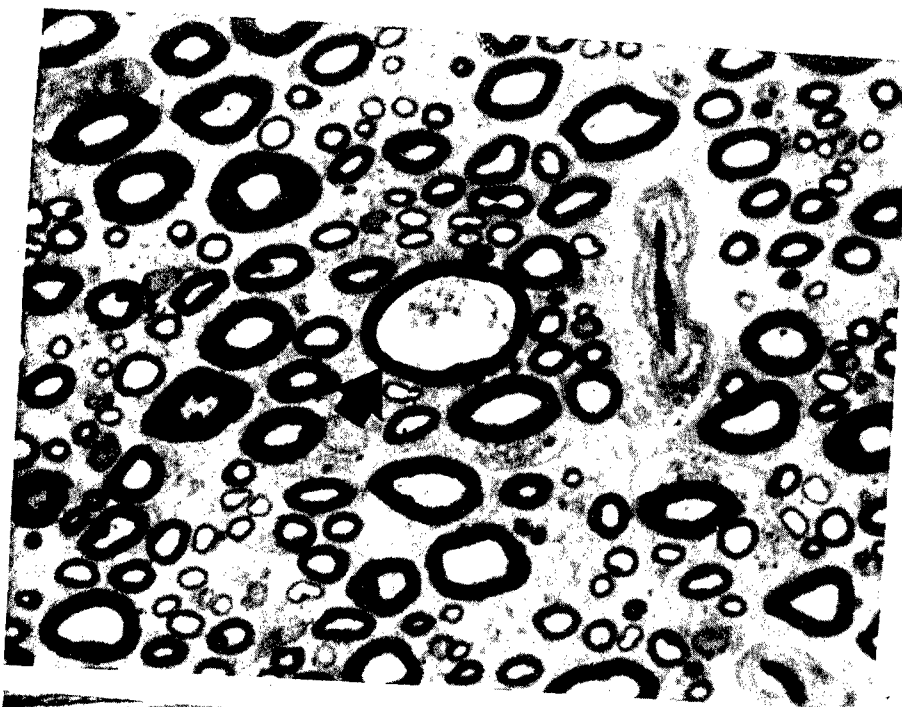


Figure 3. Nerve fiber with swollen debris containing axon (arrow) in a cross-section of the superficial fibular nerve from a chicken with DFP-induced delayed neuropathy. Toluidine blue stain, x1320.

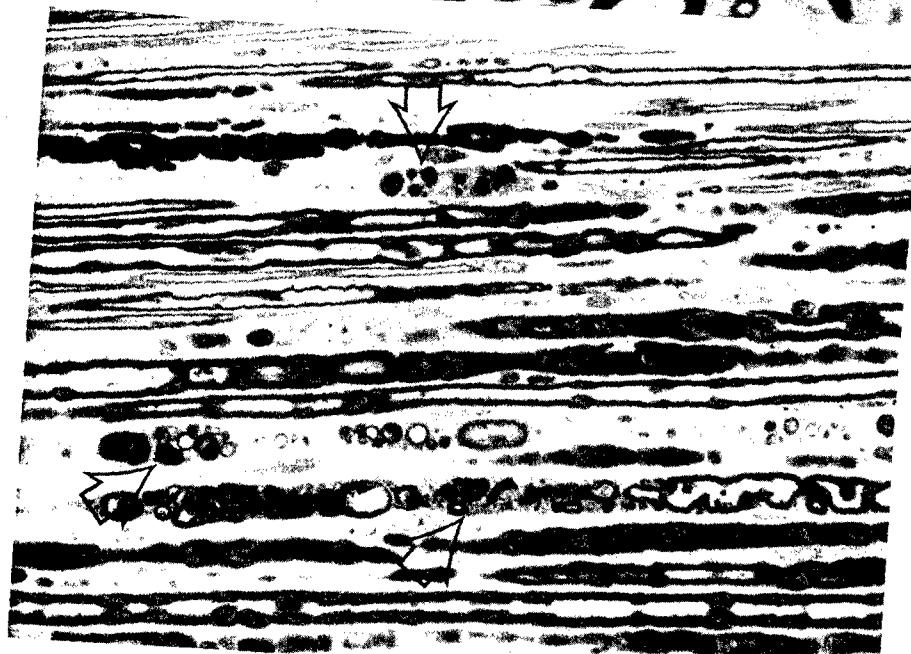


Figure 4. Longitudinal section of the superficial fibular nerve of a chicken with TOTP-induced delayed neuropathy. Several nerve fibers undergoing wallerian-like degeneration are present (arrows). Toluidine blue stain, x500.

Regeneration in the form of lateral and ultraterminal branching from the intact portion of peripheral nerve fibers whose motor terminals had degenerated due to TOTP or DFP delayed neuropathy has been seen (Cavanagh, 1964; Glazer et al., 1978). Such changes are noted weeks after the clinical onset, at a time when clinical deficits no longer progress, and some improvement is seen.

ELECTRON MICROSCOPY AND TEASED FIBER STUDIES

Organophosphorus induced delayed neurotoxicity has elicited lesions classified as having focal axonal alterations at the commencement of nerve fiber degeneration (Cavanagh, 1979). In

contrast to compounds such as hexacarbons and acrylamide, which elicit accumulation of intra-axonal filaments, neurotoxic organophosphorus compounds induce formation of membranous structures and vacuoles as focal axonal alterations preceding nerve fiber degeneration.

Several transmission electron microscopic studies of delayed neuropathy have revealed excess accumulation of branching cisternal membranous structures, resembling agranular reticulum, in the axoplasm, prior to swelling and further degeneration of the nerve fiber (Bischoff, 1967, 1970; Bouldin and Cavanagh, 1979b; LeVay et al., 1971; Prineas, 1969) (Figure 5). Where marked aggregations of such cisternal structures were present, they formed stacks or tubular arrays (Bouldin and Cavanagh, 1979b) (Figure 5). Later stages in axonal degeneration were characterized by loss of neurotubules and neurofilaments, rarification of axoplasm, the presence of degenerating mitochondria, membranous structures, and dense bodies, along with granular transformation of axoplasm (Bischoff, 1967, 1970; Bouldin and Cavanagh, 1979b; Prineas, 1969). Subsequent wallerian-like degeneration of the nerve fiber leads to the presence of axonal and myelin debris in Schwann cells (Prineas, 1969) (Figure 6).

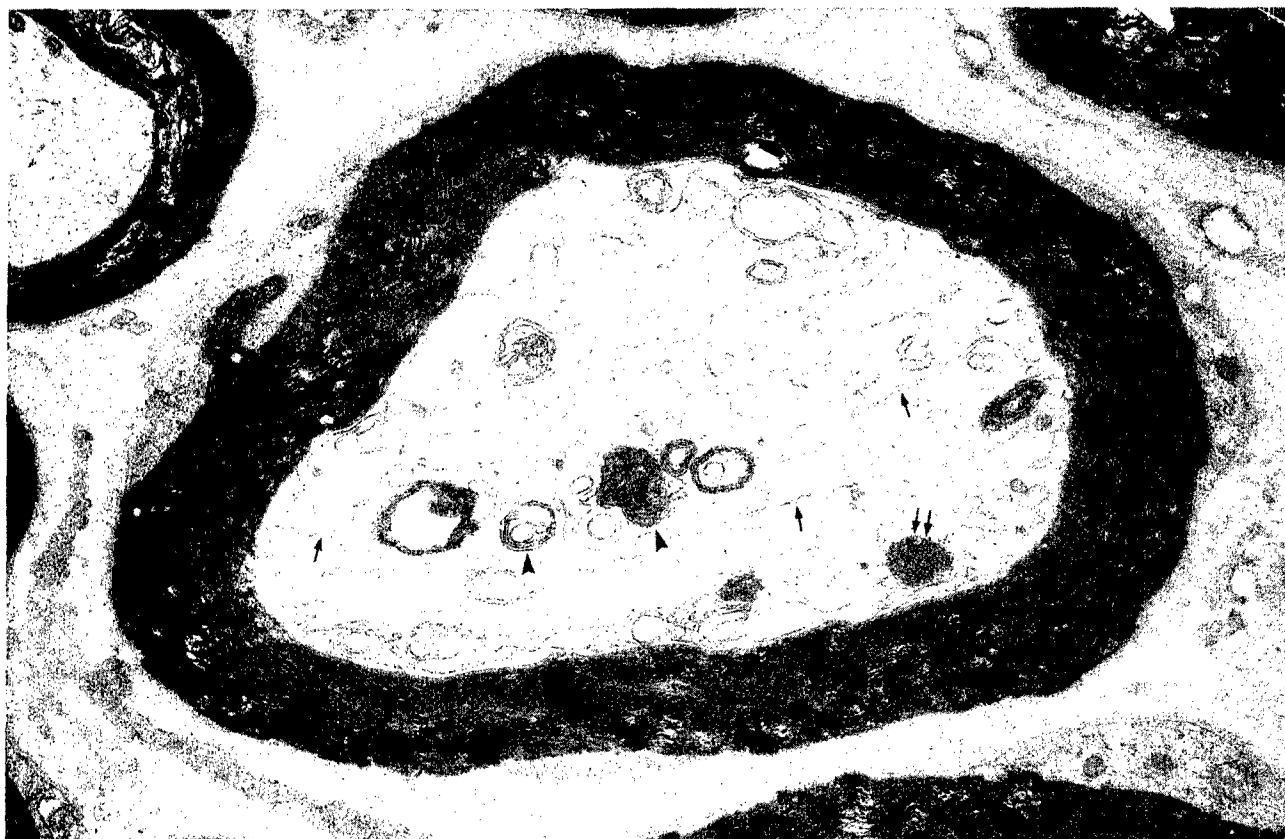


Figure 5. Cross-section of a nerve fiber of a chicken with DFP-induced delayed neuropathy. The axoplasm has increased numbers of narrow cisternal structures (arrows), membranous whorls (arrowheads) and tubular arrays (double arrows). Superficial fibular nerve, x11900.

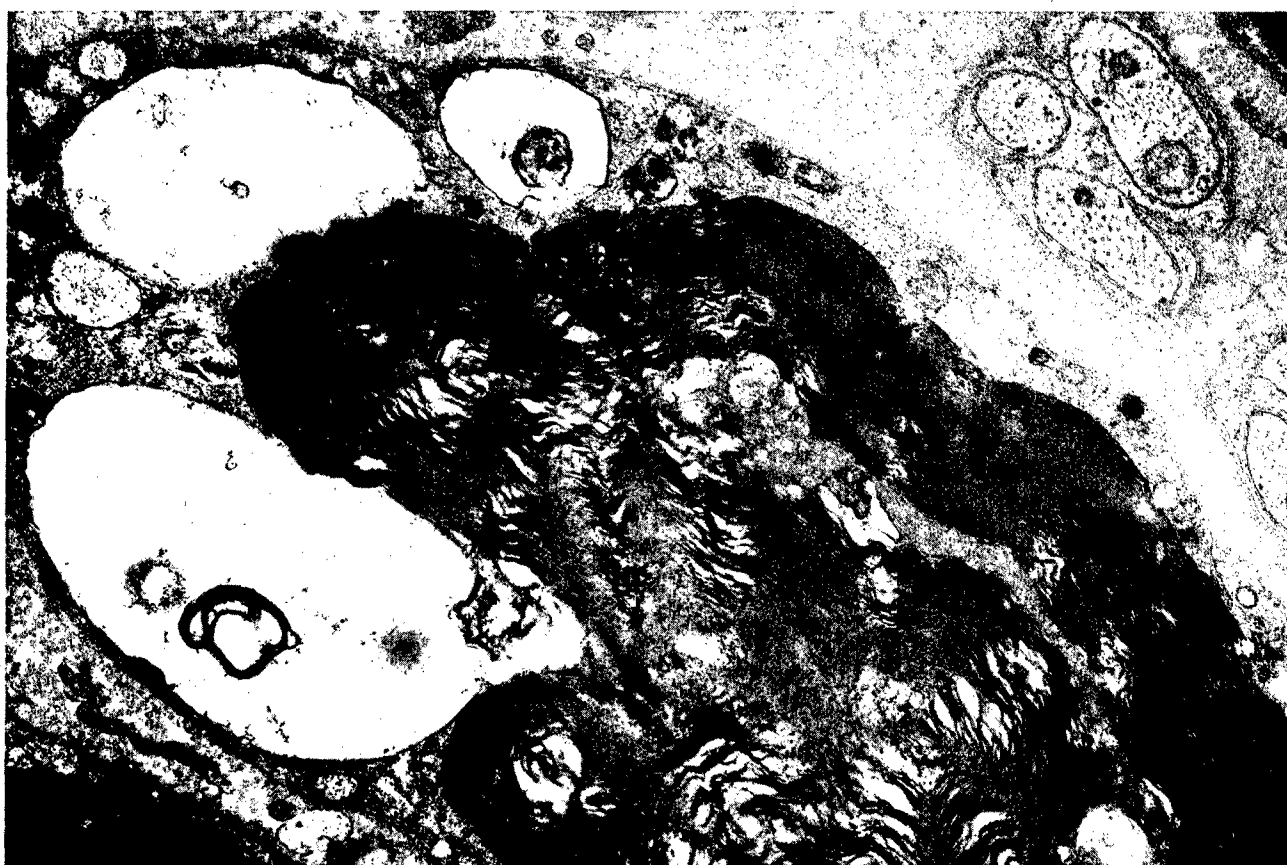


Figure 6. Schwann cell containing myelin and axonal debris in a chicken with DFP-induced delayed neuropathy. Superficial fibular nerve, x20260.

The ultrastructure of axon terminals in degenerating nerve fibers has been examined (Drakontides et al., 1982; Glazer et al., 1978; Prineas, 1969). Motor terminals and spinal cord synapses were shown to be swollen and contain concentric multilamellar membranous arrays. In the neuromuscular junctions, severely affected terminals were retracted from the junction. Post-junctional membranes had changes resembling those of denervation.

A combined teased fiber-transmission electron microscopic study of the recurrent laryngeal nerve in cats with DFP-induced delayed neuropathy revealed spatio-temporal changes in the process of nerve fiber degeneration (Bouldin and Cavanagh, 1979a,b). Initial lesions, first observed 14 days after DFP administration, consisted of focal, distal but non-terminal, midinternodal, sometimes multiple nerve fiber varicosities, and paranodal demyelination. The varicosities were associated with large intra-axonal and/or intra-myelinic vacuoles. These varicosities were thought to evolve into focal, non-terminal axonal degeneration ("chemical transection"), which led to wallerian-like degeneration of more

distal portions of the fiber. A proposed mechanism for formation of vacuoles of the varicosities invoked DFP-induced breakdown of membrane controls regulating intracellular-extracellular ionic gradients. This would result in passive influx of water into intra-axonal space and intraperiod gap of the myelin sheath.

PROPOSED CELLULAR PATHOGENETIC MECHANISMS

As noted elsewhere in these proceedings, there appears to be some understanding of initiating events in organophosphate-induced delayed neurotoxicity, leading to inhibition and then "aging" of nervous system neurotoxic esterase (Johnson, 1982). However, the process by which subsequent axonal injury occurs, with development of the neurodegenerative changes described above, has not been definitively determined. Recent studies have suggested several possible pathogenetic mechanisms, some of which are considered below.

Since the largest, longest, nerve fibers are affected, the suggestion that the amount of work required in cell maintenance is a factor in organophosphorus-induced delayed neuropathy has been made (Cavanagh, 1974). This might relate to interference with synthesis, transport, or utilization of essential materials. In a similar vein, the possibility that neurotoxic organophosphorus compounds might interfere with a trophic substance needed for nerve cell preservation has been suggested (Glazer et al., 1978).

The cell bodies of affected axons show no significant primary morphologic changes, and there appears to be a potential for regeneration from intact distal portions of the injured axon (Cavanagh, 1964; Glazer et al., 1978; Reichert and Abou-Donia, 1980). It would therefore seem that significant perikaryal injury was not a major factor in organophosphorus delayed neuropathy. The distal, multifocal, early sites of axonal injury would also argue against this possibility (Bouldin and Cavanagh, 1979a).

Alteration of some facet of axonal transport is a possible factor. Data in this area of investigation are somewhat inconsistent. Studies showing either significant or minimal effects on components of axonal transport in various models of organophosphorus-induced delayed neuropathy have been published (Bradley and Williams, 1973; James and Austin, 1970; Pleasure et al., 1969; Reichert and Abou-Donia, 1980). The possibility of a localized disruption of axonal transport has been raised (Reichert and Abou-Donia, 1980).

Organophosphorus-induced membrane injury in nerve fibers may be involved. A suggested breakdown of membrane control of intracellular-extracellular ionic gradients leading to early water

influx of nerve fibers, with subsequent wallerian-like degeneration, is worthy of further consideration (Bouldin and Cavanagh, 1979a,b). A role for alteration of lysosomes in the neurodegenerative process has also been suggested (Abou-Donia, 1981). Clearly, additional investigation of the pathogenetic sequence of nerve fiber injury in organophosphorus induced delayed neuropathy is needed.

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**ELECTROPHYSIOLOGIC CHANGES IN ORGANOPHOSPHORUS-
INDUCED DELAYED NEUROTOXICITY**

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There is now a considerable body of literature showing that organophosphorus (OP) agents which produce organophosphorus-induced delayed neuropathy (OPIDN) also inhibit neurotoxic esterase (NTE). Although the functional relationship of these two events has not been established, the correlation is strong enough that NTE inhibition can be used as a reliable predictor of the peripheral neuropathy potency of a given agent (Johnson, 1975). There is a wealth of data describing the histopathology and biochemistry associated with OPIDN, but few electrophysiologic correlates have been made.

To some extent, this is understandable. All the OPs inhibit acetylcholinesterase (AChE) and plasma cholinesterase (ChE), resulting in large accumulations of acetylcholine. The increase in cholinergic transmission increases autonomic and skeletal muscle activity peripherally and many neuronal pathways centrally. Therefore, many of the electrophysiologic changes which are observed after OP exposure are really due to the increase in transmitter levels and not a direct effect of the OP. There is exhaustive literature describing the resulting neuronal responses. However, since the effects of OPs on AChE do not play a role in OPIDN, the electrophysiologic events associated with accumulating acetylcholine complicate studies directed at finding electrophysiologic correlates to OPIDN. Nevertheless, there are reports which have provided evidence that OPs have pharmacologic effects in addition to their ability to inhibit the cholinesterases.

It was noted more than 30 years ago that di-isopropylfluorophosphate (DFP), an irreversible AChE inhibitor, blocked axonal nerve conduction (Grundfest et al., 1947). There was a long debate in the literature suggesting that this may in fact be due to AChE inhibition in the axon (Feld et al., 1947; Sechzer, 1965; Hoskin et al., 1966; Kremzner and Rosenberg, 1971). However, esterases are inhibited in vitro by DFP at concentrations (10^{-8} to 10^{-6} M) much lower than those required for nerve blockade (10^{-3} to 10^{-2} M). In addition, the tri-isopropyl analogue of DFP which is not an enzyme inhibitor also blocks nerve conduction (Woodin

and Wieneke, 1970). There are many similarities between the effect of DFP and procaine (Toman et al., 1947), suggesting that at high concentrations DFP may have a nonselective detergent effect on the membrane which produces local anesthesia.

Exposure to the potent OP, soman, induced profound increases in the EEG activity of monkeys, which most likely are due to the sudden increase in central acetylcholine concentrations. However, the EEG activity progressively shifted to a postictal phase despite continued AChE inhibition. Although the author (Lipp, 1968) suggested that the depression might be due to secondary factors such as anoxia or fatigue, there is a possibility that soman was having other central pharmacologic effects. For instance, Bay et al. (1973) showed that soman uncoupled the phasic activity of the phrenic nerve without accompanying changes in blood pressure or respiratory rate. The changes in neuronal firing of pons and medullary units did not correlate with the degree of cholinesterase inhibition in these tissues. Also, VanMeter et al. (1978) showed that DFP and sarin induced changes in the EEG of rabbits despite pretreatment with physostigmine and complete inhibition of AChE. They concluded that OPs exert direct actions at CNS sites which are unrelated to AChE inhibition and acetylcholine accumulation.

Finally, there is evidence that OPs also bind to the acetylcholine receptor (Kuba et al., 1973; Bullock et al., 1977; Farquharson et al., 1980). These effects probably contribute to the acute toxicity of the OPs, and the authors did not propose that these actions contribute to OPIDN.

Nevertheless, Roberts (1979) has suggested that electromyographic measurements would be a useful and sensitive method for monitoring workers who must handle chemicals which pose an OPIDN hazard. Table 1 shows that there are a number of reports in the clinical literature showing that decreased nerve conduction velocity accompanies OPIDN. However, it should be noted that by the time these measurements were made the cases were probably in the advanced stages and the patients were exhibiting many signs of toxicity. In some cases, no nerve excitability could be found, suggesting that nerve atrophy probably had occurred.

In studies to determine whether noninvasive electrophysiologic measures could be used to monitor OPIDN, Maxwell and LeQuesne (1982) found that chronic exposure to OPs for up to three months produced no change in the muscle action potential or motor nerve conduction velocity of rats. They concluded that conduction velocity and measures of muscle activity which were made in a clinically acceptable manner were not good predictors of OP toxicity. However, since rats are resistant to chronic OP exposure and no behavioral deficits were noted, it would have been difficult to demonstrate electrophysiologic changes. No signs of OPIDN were noted in this study.

TABLE 1. CLINICAL REPORTS OF CHANGES IN CONDUCTION FOLLOWING EXPOSURE TO ORGANOPHOSPHORUS AGENTS

<u>Decreases Reported</u>	
Tri-orthocresylphosphate Dipterex	Vasilescu, 1972
Diethyl 4-nitrophenyl phosphorothionate Dimethyl 4-nitrophenyl phosphorothionate 2-chloro-1-(2,4-dichlorophenyl)-vinyl diethyl phosphate O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)- phosphorothionate	Roberts, 1976
Bromophos Diazinon Dursbane Malathion	Stalberg et al., 1978
Dipterex	Hierons & Johnson, 1978
Tri-orthocresylphosphate Dipterex Divipan	Vasilescu & Florescu, 1980
<u>No Change Reported</u>	
Thimet Avisol-DM	Jusic et al., 1980
Phytosol	Jedrzejowska et al., 1980
Metrifonate	Maxwell et al., 1981

In contrast to noninvasive electrophysiologic techniques which provide a very insensitive measure of nerve function, invasive techniques in animals have a much greater chance of detecting subtle changes in nerve activity following OP exposure. In experiments which examined the effect of OPs on neuromuscular function, the effects of dichlorvos on tetanic and posttetanic stimulation were blocked by pretreatment with physostigmine (Owczarczyk et al., 1980). Each OP inhibited AChE by about 50% and the combination produced no further enzyme inhibition, suggesting the protective effect was not due to accumulation of acetylcholine. Since physostigmine also prevents OPIDN induced by agents such as dichlorvos, it is interesting to speculate that this acute effect at the nerve terminal might be an early sign of OPIDN.

Lowndes and coworkers have followed the effects of DFP on nerve terminal function for several weeks after an acute exposure restricted to one hindlimb in the cat. Simultaneously with the onset of behavioral changes in motor control of the affected hindlimb, there was a progressive loss of posttetanic potentiation in the soleus muscle. The concurrent loss of posttetanic repetitive activity in the sciatic nerve indicated that the DFP-induced deficit was at the motor nerve terminal (Lowndes et al., 1974; Lowndes et al., 1975). Muscle spindle afferents show a loss of responsiveness over the same time course (Baker and Lowndes, 1980). By pretreating the cats with phenylmethanesulfonyl fluoride (PMSF), which is a protective inhibitor of NTE (Johnson, 1970), the effects of DFP on the motor nerve terminal could be prevented (Baker et al., 1980), providing further proof that the nerve terminal is an early site of OPIDN. However, since the administration of DFP in these experiments was restricted to the hindlimb and did not circulate systemically (Howland et al., 1980), one can argue that this may not be the most sensitive site of OP intoxication in the nervous system.

Following systemic injection of tri-orthocresylphosphate (TOCP) to cats there were significant reductions in the size of the unconditioned spinal cord monosynaptic potential and post-tetanic response (Lapadula et al., 1982), suggesting a reduction in both the discharge zone and subliminal fringe.

It is important to note that in these studies of motor and sensory axons and spinal cord activity, there were no concurrent changes in conduction velocity. This is consistent with a number of clinical reports (see Table 1) in which patients had normal conduction velocities despite obvious signs of OPIDN. However, these observations are not consistent with histopathology studies which have shown that long, large diameter axons are most vulnerable to OPIDN (Spencer and Schaumburg, 1978). The electrophysiologic studies suggest that the large diameter axons, which are also fast conducting, are preserved in the early stages of OPIDN. The only way to reconcile these two observations is to invoke the unconventional notion that as the large fibers drop out, the remaining nerves compensate via some homeostatic mechanism by conducting more rapidly.

There are some recent observations which tend to support this view. Repetitive activity in evoked muscle action potentials has been reported following chronic OP dosing in rats (Maxwell and LeQuesne, 1982). Tests were not performed to determine whether this was due to an effect directly in the muscle or was the result of enhanced neuronal input.

Our own experiments have shown that OPs have excitatory actions on peripheral nerve membrane. Chronic dosing of tri-chlorfon to rats resulted in a progressive shift in the relative refractory period (Averbook and Anderson, 1983). Subsequently, we have noted a similar effect following DFP and also soman administration. For all three agents, the refractory period was shortened, thus making the nerve more excitable and capable of carrying higher frequency activity. Despite continued dosing, however, the relative refractory period was eventually restored to the normal range. Figure 1 shows that the time course of these changes in refractory period roughly correlates with the

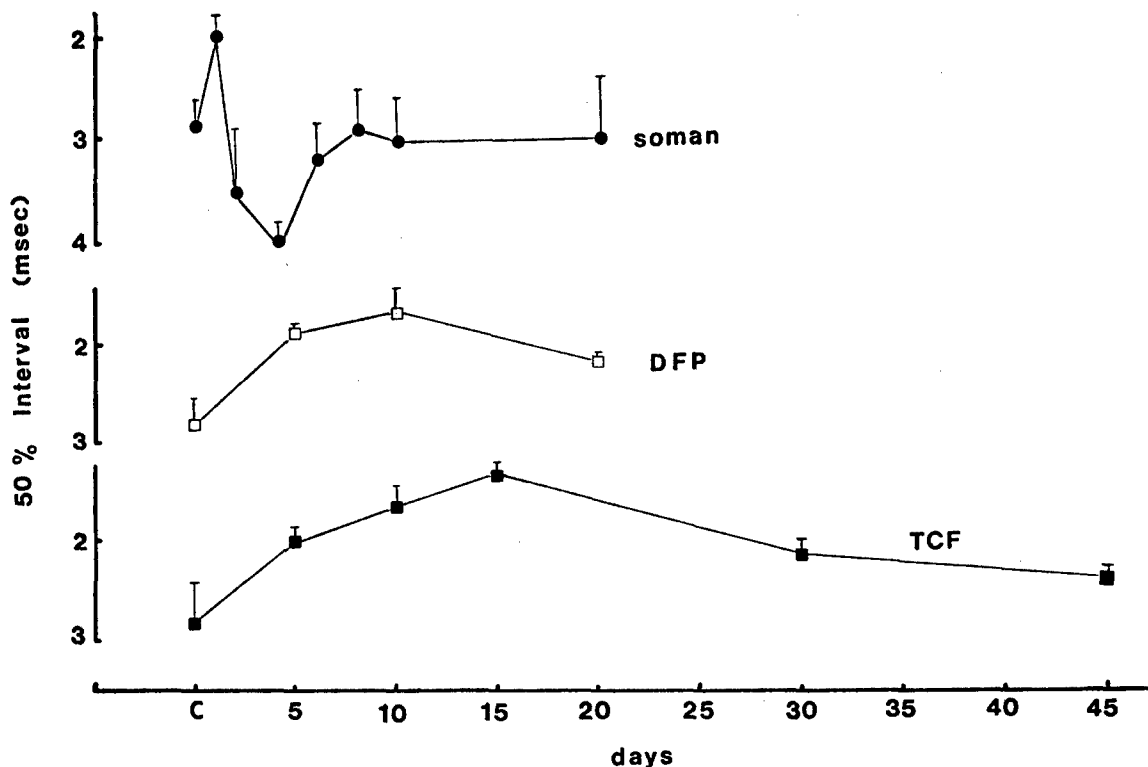


Figure 1. Effect of organophosphorus agents on the relative refractory period of sciatic nerve. Rats were dosed with either soman (0.05 mg/kg), DFP (1 mg/kg), or trichlorfon (200 mg/kg) I.P. daily for up to 45 days. At the intervals indicated, rats in groups of five were withdrawn from the study and the sciatic nerve was exposed for examination in situ. Each point represents the mean (\pm SE) interstimulus interval which produced a 50% reduction in the amplitude of the conditioned compound action potential response (for details of method, see Averbook and Anderson, 1983). Initial dosing with each agent produced a shortening of the relative refractory period. Continued dosing produced an attenuated effect, and the relative refractory period gradually returned to control.

potency of the compounds. Soman, the most potent agent, induces rapid shifts in the refractory period; trichlorfon, a very weak agent, shows much more gradual changes in nerve refractoriness. It is important to note that (1) these changes do not correlate with AChE inhibition (Anderson et al., 1983) and (2) that parathion, an OP agent which does not produce OPIDN, will not produce these changes in refractory period, despite considerable inhibition of AChE (Averbook and Anderson, 1983).

These changes may be a reflection of homeostatic mechanisms taking place in the nerve. In the face of OP intoxication and the loss of axons or functioning nerve terminals from the nerve bundle, the remaining axons compensate by becoming more responsive. The rat is capable of overcoming the OP insult, which is reflected in (1) the restoration of the relative refractory period, (2) the observation that the animals show no behavioral deficits, and (3) these animals do not develop OPIDN. Susceptible species, on the other hand, presumably cannot make all the critical adjustments in excitable membrane function. The OP in these species predominates and OPIDN results. Further investigations along these lines, in both susceptible and nonsusceptible species, could provide useful information to establish the connection among the biochemical, pathological, and physiologic factors contributing to OPIDN.

There are many gaps in our present understanding of the changes in neuronal excitability which may be induced by OPs and contribute to the development of OPIDN. The present body of literature, however, has provided some interesting observations which indicate that electrophysiologic measurements may enhance our understanding of the mechanisms responsible for OPIDN.

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BIOCHEMISTRY AND PATHOGENIC HYPOTHESES
OF
ORGANOPHOSPHORUS-INDUCED DELAYED NEUROTOXICITY

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The clinical and pathological aspects of organophosphorus delayed neuropathy have been previously detailed (Sprague, 1984; Jortner, 1984). This report will be a brief review of the investigations which provide data supportive of the hypothesis that a membrane-associated protein is the initial target of neurotoxic organophosphorus compounds (the term "neurotoxic OP" will be used in this paper to refer to organophosphorus compounds which produce delayed neuropathy). Findings from more recent studies will be used to develop a "picture" of the molecular state of the protein at the outset of the neurotoxic process.

Early reports from Smith et al. (1930), Bidstrup et al. (1953), and Barnes and Denz (1953) indicated that certain aryl phosphates, alkyl phosphorodiamidofluoridates, and alkyl phosphorofluoridates, respectively, produced delayed neuropathy in humans and laboratory animals. Poulsen and Aldridge (1964) noted that these molecular structures were of the sort known to phosphorylate proteins, proteins which normally had hydrolytic enzyme activity. Indeed, many neurotoxic OPs were inhibitors of various esterases and proteases. Thus, the postulate was made that the compounds were neurotoxic by virtue of their capacity to phosphorylate and consequently inhibit the action of some hydrolytic enzyme. However, for this to be true, an enzyme had to be identified which was inhibited consistently and exclusively by neurotoxic OPs. Studies examining the inhibition characteristics of known esterases revealed no enzymes which fit the criteria (Davison, 1953; Aldridge and Barnes, 1961; 1966a,b).

In 1969, Johnson (1969a) proposed that if phosphorylation of a specific protein was required as a step in the mechanism of delayed neuropathy, the site might be labeled using a radionuclide-tagged neurotoxic OP. However, due to the fact that neurotoxic OPs phosphorylated a number of proteins, Johnson cautioned that the pertinent site might be difficult to identify amidst "background" labeling. It was hoped that one might eliminate

irrelevant binding sites by blocking them with a compound which would not affect the target protein. In theory, this could be accomplished by pretreating tissue with a non-neurotoxic organophosphorus compound before performing the radiolabeling procedure. This approach worked relatively well. Using [^{32}P]-diisopropylphosphorofluoridate ([^{32}P]-DFP) as the labeling compound, it was found that a substantial portion of bound radioactivity was excluded in hen brain homogenates preincubated with non-neurotoxic organophosphorus compounds such as tetraethyl pyrophosphate (TEPP) or paraoxon. A segment of the bound radioactivity present after TEPP blockade was eliminated if the preincubation was carried out in the presence of both TEPP and the neurotoxic OP, mipafox (N,N'-diisopropylphosphorodiamidofluoridate). Binding of [^{32}P]-DFP to a site putatively involved in organophosphorus delayed neuropathy would contribute to the radioactivity measured in this segment. That a relevant OP binding site did exist was suggested by the observation that the TEPP-resistant, mipafox-sensitive binding was substantially reduced in incubates of tissue taken from hens treated with neurotoxic OPs (Johnson, 1969a).

As previously mentioned, proteins phosphorylated by organophosphorus compounds often have hydrolytic enzyme activity. It was hypothesized that the "target protein" phosphorylated by neurotoxic OPs could be an esterase, and a search for substrates was conducted. Organophosphorus compounds generally phosphorylate an esterase at its catalytic site (Aldridge and Reiner, 1975). Thus, in a system where an esterase is concurrently incubated with a radiolabeled organophosphate and an appropriate substrate, bound radioactivity would be reduced due to competition for the catalytic site. Using this method combined with the differential labeling procedure, Johnson (1969b) found that phenyl phenylacetate (PPA) blocked phosphorylation of the target protein. Hydrolysis of PPA by enzymes in brain homogenates was surveyed by monitoring the production of phenol in an incubation system. Hydrolytic activity was diminished by preincubation of the homogenate with TEPP or paraoxon, and an additional portion of PPA-hydrolytic activity was eliminated if the homogenate was preincubated with mipafox. These characteristics were reminiscent of the differential inhibition of [^{32}P]-DFP binding to brain homogenates. Reduction of paraoxon-resistant, mipafox-sensitive hydrolase activity in brains from hens treated with organophosphorus compounds was quantitatively correlated with decreases in [^{32}P]-DFP binding to the target protein. The correlation persisted in tests with a large number of compounds, and indicated that the target protein was exhibiting esteratic activity (Johnson, 1970). The target protein has since been referred to as "Neurotoxic Esterase" or "Neuropathy Target Esterase", in either case abbreviated "NTE" (Johnson, 1984).

It has become common practice to monitor phosphorylation of NTE by noting the capacity of a compound in question to inhibit its esteratic activity. NTE activity is defined as the difference in the amount of phenyl valerate hydrolyzed by paired tissue

samples preincubated with paraoxon alone and paraoxon plus mipafox. Phenyl valerate is used because it appears to be a more suitable substrate than phenyl phenylacetate (Johnson, 1975).

During a screen of non-organophosphorus compounds for inhibition of NTE activity, it was found that phenyl benzyl carbamate and phenyl methanesulfonyl fluoride inhibited NTE but did not produce neuropathy (Johnson and Lauwerys, 1969; Johnson, 1970). Later, it was found that certain phosphinates also would inhibit NTE without producing neuropathy. These observations eliminated the possibility that inhibition of esteratic activity was responsible for the initiation of organophosphorus delayed neuropathy. However, it was found that non-neurotoxic inhibitors of NTE would protect against the toxicity of subsequently administered neurotoxic OPs. Presumably, the protective effect was derived from competitive blockade of the NTE binding site. The important implication of this observation was that NTE phosphorylation was not merely correlated with delayed neuropathy; rather, it was a necessary step in its pathogenesis.

To reiterate, covalent binding to the catalytic site of NTE appeared to be a necessary step in the pathogenesis of organophosphorus delayed neuropathy. However, the observation that carbamylation, sulfonylation, or phosphinylation of the catalytic site did not produce neurotoxicity indicated that simple covalent binding to the catalytic site of NTE was not sufficient. It was therefore pertinent to consider what neurotoxic OPs did following binding (or in addition to binding) which distinguished them from non-neurotoxic compounds.

Returning to the analogy of covalent attack on other esterases, it was likely the binding site on NTE was a serine-hydroxyl function (this has yet to be directly demonstrated). Using this assumption, one may depict the molecule following attack by various compounds as having one of the structures in Figure 1. Structures I, II, or III (where R is either an alkyl or an aryl group) are produced by initial attack of organophosphorus compounds known to be neurotoxic. Non-neurotoxic NTE "blockers" are carbamylate (structure IV), sulfonylate (V), or phosphinylate (VI) NTE. The structures which result from neurotoxic OPs have at least one side chain where something comparable to a hydrolytic cleavage (loss of an R-O or R-N group) could occur. This sort of cleavage is known to occur with other esterases, and is referred to as aging (Aldridge and Reiner, 1975b). Clothier and Johnson (1979, 1980) demonstrated that a similar (but not identical) reaction occurs with neurotoxic OPs following their binding to NTE. Structures IV, V, and VI are not subject to such a reaction. It was therefore proposed that covalent binding at the catalytic site followed by aging was a necessary step in the pathogenesis of organophosphorus delayed neuropathy (Johnson, 1974; Clothier and Johnson, 1979, 1980).

the protein (Clothier and Johnson, 1980; Williams, 1983). This was in contrast to reports on cholinesterases where the leaving group diffused away in the incubation medium (Michel et al., 1967). In sum, phosphorylated, aged NTE possesses a negatively charged phosphoryl moiety attached at its catalytic center, and an alkyl- or aryl group covalently bound to a different site, presumably nearby (Figure 2).

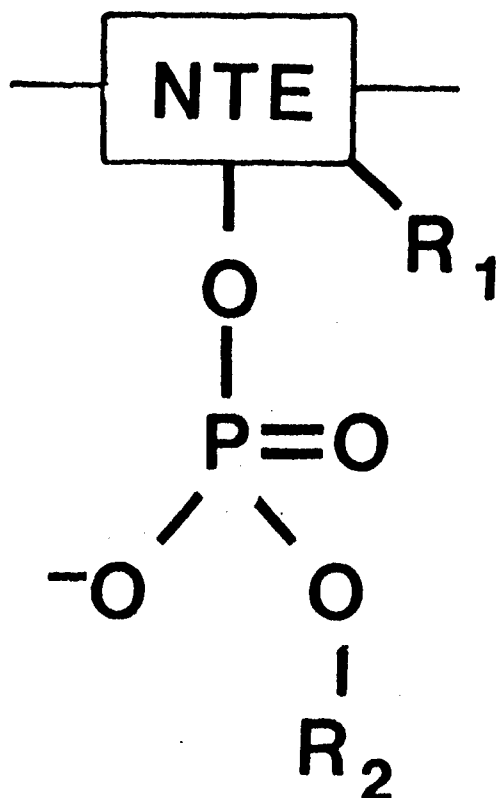


Figure 2. Schematic drawing of phosphorylated, aged NTE.

The role played by alterations of the molecular state of NTE in the pathogenesis of organophosphorus delayed neuropathy may only be hypothesized. However, several facts concerning NTE and delayed neuropathy influence speculation.

Organophosphorylation and subsequent aging of NTE is a relatively rapid process. Although the pharmacokinetics of individual neurotoxic OPs might vary, it is likely that with almost any compound the aged moiety can be generated within 24 hours of exposure. Yet frank pathological or clinical signs of neuropathy are not evident for at least 8 days, and, more commonly, take longer. This observation must be addressed in any hypothesis of

neurotoxic mechanism. For example, NTE phosphorylation might be considered an early event in a long, multi-step process, or a single event which causes persistent and cumulative "damage" until the condition becomes overt (Johnson, 1982; Richardson, 1983).

A majority of the work described above was done using hen brain as the source of NTE. However, relative to peripheral nerve and long spinal pathways, minimal pathological change is found in brain. NTE is present in spinal cord and peripheral nerve (Caroldi and Lotti, 1982) in concentrations less than that of brain (Dudek and Richardson, 1982). NTE has been tentatively identified in other tissues including spleen, lymphocytes (Dudek and Richardson, 1982), placenta (Gurba and Richardson, 1983), and liver and kidney (Williams, 1983). Hypotheses implicating NTE in the pathogenesis of organophosphorus delayed neuropathy must account for the specificity of effect despite disseminated presence of the protein. It must also be considered whether or not action of neurotoxic OPs on NTE in non-neuro tissue plays a role in neurotoxicity. Moreover, there are species differences in susceptibility to organophosphorus delayed neuropathy; hen, cat, and man are sensitive to the point that a single dose of a neurotoxic OP will produce neuropathy, but rats require large, multiple doses (Johnson, 1982). NTE is present in these and all species studied, although in differing concentrations (Gurba et al., 1981).

The lipophilicity of many substrates and inhibitors of NTE indicates that the active site of NTE is hydrophobic or obscured in a biomembrane. Examination of the subcellular distribution of NTE in hen brain homogenates reveals that at least 96% of the enzyme activity is membrane bound, and nearly 70% pellets in the "microsomal" fraction along with 5'-nucleotidase, a plasma membrane marker (Richardson et al., 1979). Enzyme activity in microsomes can be solubilized, but retention of activity in a soluble fraction requires the continued presence of detergent (Davis et al., 1980). These results are consistent with NTE being a membrane-associated protein and its hydrolytic activity being membrane-dependent. It is therefore pertinent to consider what perturbations in membrane structure and function might be brought about by phosphorylation and aging of NTE. A conformational change in NTE might result from the negative charge or alkylation/arylation of the molecule. Such an alteration could lead to membrane dysfunction if NTE plays any role in maintaining membrane integrity. Alternatively, negative charge generated by aging of NTE might accumulate and upset the normal charge balance at the plasma membrane. Refining these hypotheses and ultimately elucidating the role of NTE in organophosphorus delayed neuropathy and normal physiology will require a greater understanding of the physical biochemistry of NTE and its relationship to membrane dynamics.

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CRITICAL OVERVIEW
OF
HEXACARBONS AND ORGANOPHOSPHATES

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At the outset it seems worthwhile to compare and contrast the two groups of agents that have been discussed today - the neurotoxic hexacarbons and the delayed neurotoxicity of organophosphates. Dr. Spencer indicated that distal axonal degeneration is by far the most common pathologic manifestation of chemical neurotoxicity in general. The groups of agents under discussion both produce types of distal axonal degeneration. These agents have received extensive study, not only because of their public health implications, but as model systems for this type of neuropathology. In the last few years a wealth of basic toxicology, biochemistry, and data describing structure-function relationships has developed around both groups of agents. Parenthetically, it is difficult to overestimate the value of this broad research effort. I have been interested in what has until recently been an "orphan" toxin, B,B,'-iminodipropionitrile or IDPN, about which we know very little concerning metabolism, biotransformation, and pharmacokinetics. Some aspects of its toxicity have been examined in detail; at the present time basic data on metabolism and the toxicity of metabolites are requirements for further insights into the mechanisms of action of IDPN.

Not only do both of the groups of agents under discussion produce axonal degeneration, but they appear to act directly on the axon, rather than requiring participation of the nerve cell body. Intraarterial injections of organophosphates into the femoral artery have produced degeneration of the distal sciatic nerve complex of cats. Epineurial or endoneurial administration of 2,5-hexanedione have produced characteristic axonal changes at the site of application.

Among the differences between these groups of agents, the early ultrastructural pathology requires comments. As we have heard, the neurotoxic hexacarbons produce accumulations of neurofilaments within the axon, followed by distal Wallerian-like degeneration. The organophosphates produce sparser early pathology; that which has been best described is accumulation of smooth membrane-bound tubular and vesicular structures. I will return to the significance of these differing patterns of pathology in a moment.

Perhaps the most substantial difference in the current understanding of these agents, alluded to by Dr. Richardson, Dr. Spencer, and quite explicitly by Dr. Schwab, is that with the neurofilamentous axonopathies such as hexacarbon neuropathy the pathogenesis of structural changes in the nerve is understood perhaps as well as in any type of experimental neurologic disease. With the organophosphates we are at the other extreme. The gap between biochemical changes and alterations in nerve structure is yet to be bridged. Several reasons may contribute to this difference between the agents. A minor factor may be the fact that so much of what we know about organophosphate neurotoxicity comes from studies of the hen. To some extent this unusual experimental animal may have led to creation of a separate literature which is not widely read by pathologists, physiologists, and biochemists, used to working with small laboratory mammals. That gap can readily be bridged both by inducing the disease in mammals for comparative studies, and by better exchange of information between those who work with fowl and those who work with mammals. A more fundamental reason for the difference in understanding these groups of agents lies in the nature of the pathology. Considering the special characteristics of neurofilaments within nerve cells, in retrospect the neurofilamentous disorders seem ideally designed for exploration and understanding of pathogenetic mechanisms. Neurofilaments are made exclusively in the cell body, and they appear to undergo transport down the axon as the assembled neurofilament organelle, rather than the soluble subunits. They move in a coherent fashion at a rate of about 1mm per day, and undergo little degradation enroute. Instead, they appear to undergo rapid proteolysis at the nerve terminal. An organelle with this life history can be followed and "tracked" by axonal transport studies, and visualized in electron micrographs. The relationship of axonal transport changes to structural alterations is straightforward. In hexacarbon neurotoxicity the sequence that Dr. DeCaprio and Dr. Sahenk listed can be summarized as follows: several agents that produce neurofilamentous neuropathies, including the hexacarbon solvents and IDPN, appear to in some way interact with or influence the axonal cytoskeleton. This can result in an easily recognizable reorganization of the axonal cytoskeleton, as Dr. Sahenk illustrated. The consequence of this reorganization appears to be a profound block in the ability of neurofilaments to move down the nerve fiber. That has been known with IDPN for some time and now has been shown to be the case with the potent analogue of 2,5-hexanedione, 3,4-dimethyl 2,5-hexanedione (DMHD). A single high dose of these agents stops the movement of neurofilament proteins all along the axon, and the consequence is the accumulation of neurofilament proteins at some point in the nerve fiber. Thus, in these disorders it has been relatively easily to reconstruct the changes in the axon and relate them to abnormalities in axonal transport. It is worth recalling at this point, however, that what remains poorly understood in the neurofilamentous neuropathies is the stimulus and mechanism underlying the late Wallerian-like degeneration of the distal axon. Dr. Sahenk presented intriguing evidence that

accumulation of rapidly transported organelles in regions of cytoskeletal disorganization may lead to axonal breakdown, but in other situations massive neurofilamentous changes may occur without any degeneration of fibers at all. Thus the relationship between neurofilamentous changes and irreversible axonal degeneration is not well understood, and it is even possible that the basis for the distal axonal degeneration in the neurofilamentous disorders is distinct from the biochemical changes which produce the neurofibrillary changes.

At this point it is appropriate to reflect briefly on what we know about Wallerian degeneration in the first place. Wallerian degeneration is a complicated and incompletely understood phenomenon. It was initially recognized in the 1850s when Waller cut peripheral nerves and documented degeneration of the distal stump, with survival and regeneration from the proximal stump. It became apparent that continuity between the cell body and the axon was necessary for survival. That observation was put on a clear biologic basis with the recognition of axonal transport, as Dr. Sahenk described. Viability of the axon clearly requires a continual delivery of the essential materials from the nerve cell body to the axon. The axon is unable to locally synthesize proteins or a variety of other important materials. For this reason there is tendency to think of Wallerian degeneration as the result of interruption of axonal flow much in the way we think of a stroke which results from interruption in the blood flow to the brain. However, it is clear that Wallerian degeneration is more complicated than simple interruption of axonal transport. For example, when fast axonal transport is interrupted pharmacologically, complete sustained block will result in axonal degeneration distal to the site of blockade. However, partial impairment of axonal transport seems to be surprisingly well tolerated. Recent evidence has demonstrated that Wallerian degeneration is not a passive "starvation" of the axon but an active, explosive, energy-requiring process. It is likely to involve the activation of proteolytic enzymes within the axon, and calcium may be involved in that activation. Thus when we think about Wallerian degeneration on a chemical or toxic basis it's appropriate to wonder about mechanisms by which membrane integrity, calcium sequestration or calcium entry into the axon might be altered. The smooth membrane systems of the axon are likely to represent major sites of calcium sequestration, and provide a potential target for toxic effects that might produce distal axonal degeneration. These smooth membrane systems are abnormal in organophosphorus neurotoxicity.

Prineas and Bouldin and Cavanagh recognized that organophosphate neurotoxicity appeared to be a "chemical transection" of the axon. Both pointed out that the most prominent predegenerative change was accumulation of smooth membrane-bound tubular and vesicular structures within the axon. These elements are carried by fast axonal transport, and it is reasonable to

suggest that their accumulation may reflect more focal impairment in this transport system. Precedence for this conclusion comes from previous studies of acrylamide, zinc pyridinethione, and p-bromophenylacetylurea, in which defective fast bidirectional transport has been demonstrated in the distal axon. The studies of axonal transport in organophosphorus neurotoxicity require repetition using contemporary techniques to be easily interpreted. Because the hen is a favorable animal for studies of organophosphorus neurotoxicity, it might be of particular value to study the ciliary ganglion system of the hen. The ciliary ganglion is innervated from the third nerve nucleus by relatively short, large caliber axons which extend unbranched through the nerve and terminate in the ciliary ganglion in a spectacular synaptic terminal called the calyx. The calyx is sufficiently large to partially surround ciliary ganglion neuron. In part for reasons of size and ease of identification it has become a standard preparation in autoradiographic and biochemical studies of fast and slow axonal transport in the laboratory of Bernard Droz and his coworkers in France. They have used this system to advantage in studies of acrylamide toxicity. If organophosphate neurotoxicity can be produced in this system, it would seem to be made to order. Initial examination of whether pathologic changes can be induced in this system would be of interest.

The story of the neurotoxic esterase, reviewed by Dr. Schwab, is an intriguing one. I wondered as I was listening whether it is clear that this material is an axonal constituent. For example, does it disappear in the distal stump of a sectioned peripheral nerve, at a time when no neural constituents remain? Is it axonally transported? By placing ligatures along the course of a peripheral nerve one might find accumulation of this material with time. This would provide an indirect approach to determining whether the material is axonally transported, and whether it is moving rapidly or slowly. Finally, it would be of interest to know if there is a proximal to distal gradient in the level of the neurotoxic esterase along the course of a long unbranched peripheral nerve. Such a gradient might provide a basis for selective vulnerability of the distal axon.

OPEN FORUM I

Dr. Spencer (Albert Einstein College of Medicine): Dr. DiVincenzo, why do certain solvents such as methylethylketone appear to potentiate the neurotoxic action of n-hexane?

Dr. DiVincenzo (Eastman Kodak Company): I have a theory on why that might take place. If you look at the metabolic scheme that I have put on the screen there is extensive omega -1 hydroxylation that's required for n-hexane metabolism. This is predominantly a microsomal enzyme action. Solvents such as methylethylketone presumably bring about an increase in cytochrome P-450 and hydroxylation reactions in general. It is conceivable, if an animal is pretreated or exposed at the same time to methylethylketone or any other inducer of metabolism, that it's likely to convert more of n-hexane to 2,5-hexanedione through this omega -1 pathway and by doing so you may have onset of neuropathy that occurs more rapidly than would normally be generated from a single exposure. This oxidative pathway ultimately gives rise to 2,5-hexanedione. This is primarily a microsomal activity and it's likely to be induced in an animal that is pretreated with phenobarbital or any other inducing agent that heightens the activities of these particular types of enzymes.

Dr. Abdel-Rahman (New Jersey Medical School): In MBK toxicity we know that MEK increases the concentration of MBK absorbed by inhalation in rats and yet within two minutes no MBK is detected in the blood. Does this also happen in exposure to hexane with MEK?

Dr. DiVincenzo: Unfortunately we did not carry out those studies but what you say is very true. When we explored the metabolism of methylethylketone we also did some human studies where we attempted to establish that MBK was absorbed through the skin. We also did some inhalation exposures with human volunteers and we found that we were unable to measure MBK in the blood at all until our exposure concentrations exceeded 100 ppm and even then this was towards the end of the 4 hour exposure. This suggests that the metabolism of MBK is very rapid. We carried out some radiolabelled studies in volunteers that ingested a very low dose of MBK and found that CO₂ was produced within minutes of ingestion once again pointing to the alpha oxidation pathway and the demethylation of MBK. I suspect when you are running an exposure with both MBK and another chemical that's going to be metabolized in similar pathways you would expect that the blood concentrations of MBK would be somewhat higher because there is competition for these enzymes. That is about the only explanation I can give for the observation that you made. I would expect that n-hexane would produce a similar phenomenon.

Dr. Spencer: Dr.DiVincenzo, what does your work tell us about the potential toxicity of non-straight chain hexanes and other alkanes?

Dr. DiVincenzo: We ran a number of studies that I didn't discuss today but which evaluated the potential for other related chemicals to produce neuropathy. We have come up with a theory that any time you expose an animal to a gamma diketone or produce one metabolically you are likely to produce neurotoxicity of this sort. These studies have not yet been published but they show that materials such as 2,5-heptanedione or 3,6-octanedione, which have the gamma diketone structures, are all metabolized to 2,5-hexanedione through a variety of pathways which are very similar to the ones that I showed today. We have not tested a gamma diketone to date which is not neurotoxic.

Mr. Ralph Wands (The Mitre Corporation): I would like to ask about the site where this metabolism to the hexanedione occurs. Does this occur predominantly in the liver and is the diketone then translocated to the peripheral nervous system or does it take place at the site of injury?

Dr. DiVincenzo: I think in this case we are dealing with both situations. The liver is, as we all know, the organ that is responsible for most metabolism in the body. I think that the liver generates 2,5-hexanedione which is more water soluble than methyl butyl ketone or n-hexane and this can be transported to other target organs. But I also believe that a number of investigators have demonstrated unequivocally that other tissues are capable of carrying out the same metabolic reactions and that they occur in nerve tissue. I think there is no question about that. Which site is more important in actually leading to the final neuropathy is difficult to say.

Mr. Ralph Wands: May I follow that up further? Since the diketone is more water soluble, how much of that gets excreted in the kidney before it creates damage?

Dr. DiVincenzo: I can't give you a quantitative answer. I could merely tell you in the studies that we did we looked at the excretion of the diketone in urine. If my memory serves correct, I think about 5% of the urinary radioactivity was present as 2,5-hexanedione.

Dr. Spencer: I suppose one of the many take-home messages from Dr. DiVincenzo's talk is that really we are dealing with normal hexane neurotoxicity, that the other hexane isomers are not neurotoxic, and in fact these have been tested and have been found to be free of such activity.

Dr. Richard Henderson (Consultant): Dr. Veronesi, did you do any follow-up studies to see whether you get recovery when you stop the dosing?

Dr. Veronesi: (U. S. Environmental Protection Agency): Yes, there was an extensive follow-up in that regard. What I did was expose cultures to n-hexane for 7 or 8 weeks until the full pathology developed and then removed the hexacarbon and returned the culture to plain nutrient fluid. I then photographically monitored the entire sequence over the next 18 weeks. Very briefly what I found is that even after taking the hexacarbon away from the tissues, the axonal swelling became even more defined and larger for several weeks. After which time the fiber, the swelling itself, began to shunt back to more normal proportions and at that point I saw a Schwann cell begin to myelinate the area where the myelin had retracted. So recovery is possible. I understand that recovery is even possible in the face of exposure, but in the study I did, recovery occurred after the toxin was removed.

Dr. Spencer: I think that I can answer that by saying that after Dr. Veronesi left our laboratory we continued looking at this question with a dimethylated 2,5-hexandione introduced by Dr. Doyle Graham and his colleagues. Following cessation of intoxication with this compound we can demonstrate not only the phenomenon of remyelination that Dr. Veronesi has just described but also actual axonal degeneration that I talked about earlier.

Dr. Richardson (University of Michigan): Dr. Veronesi, you pointed out that one difference between this organotypic system and the in vivo situation is the size and we have often in this field talked about the factors that govern the relative sensitivity of axons and it being some combination of length and diameter. Here you had very short axons and very small diameter axons undergoing the same kind of degeneration. I wonder how you account for that.

Dr. Veronesi: I accounted for it by pointing out that in culture everything is in miniature, and the cell body and its metabolic machinery, if you will, is also relative in size to that axon. In this culture system, even though the typogenesis was defined, by viewing the large diameter myelinated fibers you would be misled if you stopped just at light microscopy in just viewing the living tissue. You would agree that the dogma of selective vulnerability holds true in the organotypic culture and that it's the largest fiber that dies back and only then do smaller diameter and unmyelinated fibers become involved. If you followed the pathology by electron microscopy you would see another story because even though the large diameter fibers were definitely experiencing axonal swelling and distal degeneration, so were the unmyelinated fibers. I don't think that this offends the dogma of the hierarchy of vulnerability seen in the animals and certainly in humans. I really think it has something to do with the fact that in my culture system the Nodes of Ranvier

which are the natural constrictions along the fiber are not as constricted in tissue culture as you would see in the animal. And so, indeed if a neurofilamentous plug was developing in the animal it would come to be blocked at a natural bottleneck along the fiber, that being the Nodes of Ranvier. Since this constriction is more or less missing in organotypic culture, all the fibers would experience a generalized axonal swelling.

Dr. Richardson: Dr. DeCaprio, in your study of mechanisms that involves cross-linking of neurofilament protein, you said that would account for accumulation of neurofilaments and I was confused. Do you mean to say that you actually see aggregations of neurofilaments or do you mean that the neurofilament subunit proteins are now cross-linked to each other so that you have a net accumulation of neurofilaments?

Dr. DeCaprio (State of New York Department of Health): Yes, the ultimate result would be a cross-linking between the actual neurofilaments. At a molecular level the cross-linking would be between individual monomer proteins, presumably. The net result would be an accumulation of these structures primarily at the nodes simply because they can't physically get by that point once they are covalently cross-linked.

Dr. Spencer: One of the points that came out of the Chicago Symposium was that perhaps we should be extremely cautious in thinking about the specificity or the lack thereof of neurofilament accumulation in that we can see neurofilament accumulation in a large variety of neurodegenerative diseases, including, for example, in intoxications produced by the vincristin and colchicine, where we believe we know the specific site of action and it certainly isn't on the neurofilament but rather on the neurotubule. Coupled with that thought is the recent evidence from Jack Griffin, Doyle Graham, and from my lab that seems to suggest that perhaps one doesn't have to go through the neurofilament phase to precipitate axonal degeneration or alternatively one can dissociate spatially the appearance of the swelling from the axonal degeneration. All that is a prelude to asking; Do we in fact have any idea whether the neurofilament is a target site or could it be any other protein, perhaps including glycolytic enzymes or some other enzymes involved in energy metabolism or some other site?

Dr. DeCaprio: We don't have conclusive evidence that the neurofilament accumulation is the primary event in the subsequent axonal degeneration. I think the most compelling evidence suggests that direct reaction of the diketone with neurofilaments or some other axonal structure is probably responsible for the syndrome. At least we can see a rapid reorganization of neurofilaments after direct injection of the compound which reproduces what we see in systemic intoxication quite well.

Dr. Spencer: This appears to be a minor point but it really isn't because neurofilament accumulation underlies a host of neurodegenerative diseases that we encounter on a daily basis. Hexane is therefore being used as a probe to try to uncover the etiology of these diseases. Would Dr. Griffin care to elaborate?

Dr. Griffin (Johns Hopkins School of Medicine): I would just comment that I think the real information about what produces neurofibrillar changes in general is coming from the kinds of ideas that Dr. DeCaprio and Doyle Graham have suggested. Amine-binding and pyrrole-ring formation may be the means of molecular binding of hexacarbon compounds and the questions that are outstanding are those of what the protein which forms these kinds of adducts might be and how that contributes to subsequent degeneration. I think it is also very likely that there are a wide variety of mechanisms by which neurofilaments are able to accumulate in the axonal or nerve cells in general and situations in which the cytoskeleton is reorganized in this fashion may be only one. Situations in which microtubules are directly affected may be others. The neurofilament accumulations may also be nonspecific accompaniments of anything which impairs axonal transport nonspecifically. Acrylamide may be an example of compounds that interfere with normal nerve metabolism in even more nonspecific ways.

Dr. Crocker (University of California, Irvine): Dr. Sahenk, are there points along the course of the nerve which are preferentially susceptible to penetration by these toxic agents? For example, are these near the terminal portions of the axon or near the cell body? Does there appear to be a lesion related to that point of penetration?

Dr. Sahenk (Ohio State University): The axonal swellings are observed to be located more distally along the peripheral nerve. Yet these swellings seem to be present all along. Perhaps the frequency of swellings increases as you go along distally. I'm not aware of any study that has looked at whether the nodal areas are more susceptible to penetration of the toxin than the distal nerve terminal.

Dr. Spencer: In your summary you clearly indicated that fast axonal transport was involved, but do we have any idea whether it is a primary or secondary phenomenon? In addition to asking you to respond to that perhaps Dr. Griffin might care to respond subsequently. Please, Dr. Sahenk.

Dr. Sahenk: With this dose regimen, our studies indicate that the increased vesicles develop after the cytoskeletal alterations and the relationship of organelle accumulation to the neurofilamentous accumulation suggests that the passage of these membranous organelles is impaired and therefore this could be a

secondary phenomenon Maybe Dr. Griffin would like to comment on that but I also heard from you that if the dose could be increased without a neurofilamentous accumulation, the transport impairment would occur earlier.

Dr. Griffin: I think my impression is very much the same, Dr. Sahenk, that the impairment of fast transport that occurs with the dimethyl 2,5-hexanedione lesion and with aminodipropyl-nitrile, another agent that produces neurofilamentous changes, aminodipropyl-nitrile is very modest, at least initially, and what you see morphologically is this excessive retention of material in the neurofilamentous regions. I think that we can say that neurofilamentous accumulations can produce secondary changes in fast transport. Whether there may be an additional direct effect on fast transport particularly in distal nerve regions hasn't been addressed in any kind of systematic fashion.

Dr. Thomas (Consultant): Has anybody identified the water soluble fraction in the hexane series which seems to be the culprit for neurotoxicity?

Dr. DiVincenzo: I think there is no question of the fact that hexanedione is the responsible agent. But if I understand your question I think you are dealing with a more morphological aspect. Is that correct? Perhaps Dr. DeCaprio could comment on that?

Dr. DeCaprio: In terms of the ultimate site of binding of this compound, we have detected the pyrrole adduct in tissue protein after dosing with 2,5-hexanedione. That's as far as we have gotten to the present time. We are currently trying to isolate it in cytoskeletal proteins from axons to see if this phenomenon is occurring within the axon. From that point we may get evidence for one of the various ways in which this adduct could lead to eventual nerve degeneration.

Dr. Hollingshaus (American Cyanamid Company): In many of these pathology studies it appears that there is a constriction in the axon. Is there a mechanism in axons that is responsible for this constriction aspect? It looks to me something like cytokinesis and cell division. Is there a biochemical mechanism that is responsible for that actual constriction in the axon that then appears to result in the backup of various products moving up and down the axon?

Dr. Spencer: Are you referring to the Node of Ranvier where the axon is relatively attenuated compared with the internodal regions?

Dr. Hollingshaus: In many of the micrographs there was a swelling.

Dr. Spencer: The swelling seemed to occur often in close relationship to this attenuated region that we refer to as the nodal axon. Precisely what controls the diameter of that part of the axon is unknown. There are several features which may contribute to that particular organization. For example, in terms of the connective tissue around the outside of the nerve fiber, there are different layers of connective tissue in that particular region. There are circularly disposed fibers around the Node of Ranvier which may possibly tend to constrict the axon in that region whereas, by contrast, in the internodal region it's more associated with longitudinal fibers. In addition, the axon membrane in that position is associated with a soft axillimal coating which has very specific properties and may conceivably be concerned with structural integrity. There is a paucity of neurofilaments normally within the nodal axon. That would certainly correlate with the rows of attenuation in that region. There are many features, therefore, of the node which could be correlated with this phenomenon but precisely what is responsible for that is still unknown.

Dr. Yang (NEIHS, NTP): I have a question in regard to Dr. Veronesi's lecture this morning. There was a slide comparing in vitro and in vivo metabolite identification. On the slide she demonstrated that the in vitro and in vivo metabolites of n-hexane were very similar between the neuromuscular explant and the in vivo situation. Of course the n-hexane toxicity is intimately associated with metabolism. I was wondering, in view of your comparison study, if the liver actually doesn't have much importance in the n-hexane toxicity development because the toxic metabolite is actually generated at the site of action? Namely in the neural tissue.

Dr. Veronesi: I can go back a bit and discuss the material and the methods of setting up this culture system. One of the ingredients in the nutrient fluids that continually bathed the growing culture and into which the individual hexacarbons were dissolved was the chick embryo extract. I might have glossed over that during my presentation. To obtain chick embryo extract involves literally going to a farm and ordering a couple dozen fertilized eggs and bringing those eggs back into the sterile room, cracking them open under aseptic conditions and removing the actual developing chick embryo. The embryos are mashed up and dissolved in the cocktail that I described. This has been given as an explanation as to how the actual hepatic tissue that was furnished by the chick embryos may have been enough to be able to convert the applied hexacarbons to that rather defined range of natural metabolites. We also did an experiment where just the hexacarbon was added to the nutrient fluid, put back into the incubator without the nerve muscular explants and it was able to convert, I think it was, the n-MBK to 5-hydroxy-2-hexanone. This indicated that the oxidation/reduction reactions that are necessary to convert the parent compounds take place primarily in the nutrient fluid itself.

Dr. Spencer: The compound was 2,5-hexanediol to the oxidated state 2,5-HD. While that may indeed be the explanation for the phenomenon in culture, a very fascinating question pertaining to yours is whether or not nerve tissue does have the capacity to metabolize these compounds themselves. I hope I have persuaded Dr. Richardson to comment on that.

Dr. Richardson: The comment I would have made is what you just stated. We have not worked on this question in our lab but I know that it is a question that has occurred to many people working in toxicology. The question of extrahepatic metabolism or specifically in neurotoxicology is if there is capacity for significant oxidative metabolism within brain tissue. When you think of some compounds that are presumably generated in the liver and then must be transported through the blood as fairly reactive intermediates and must reach remote sites of action where they then react in nervous tissue, you often wonder how this is possible. We don't know for certain the answer to that question although when you measure the P450 level in brain you find that it is extremely low. There is this question about reactive intermediates reaching sites of action distant from the liver when on the way they encounter all kinds of macromolecules for binding. Dr. DiVincenzo, who does toxicokinetic studies, might want to say something about this.

Dr. DiVincenzo: I believe Bus and his co-workers did some crucial experiments that suggested that metabolism for n-hexane is taking place within other tissues, particularly nerve tissue in this case. It's difficult though to ascertain exactly what role the liver may play in the formation of 2,5-hexanedione and its dispersal to other tissues with regard to this type of toxicity. However, I think there is very little question that other tissues can metabolize these materials and certainly that plays a fairly important role in the eventual neurotoxicity that occurs.

Dr. Abdel-Rahman: In the 2,5-hexanedione study the bulk of the 2,5-hexanedione was excreted and conjugated with glucuronic acid. At the same time, neuropathy was observed. Do you have any comment, Dr. DiVincenzo?

Dr. DiVincenzo: Would you be kind enough to repeat the question? I'm afraid I didn't quite catch it.

Dr. Abdel-Rahman: Dr. DiVincenzo, some of the 2,5-hexanedione was excreted without change but about 95% of 2,5-hexanedione excreted was bound to glucuronic acid or conjugated by glucuronic acid and yet you can still see neuropathy occurring in guinea pigs and rats. It was really important to know that the conjugated glucuronic acid was conjugated with the diketone. We thought that maybe this would be hexanol but it really was 2,5-hexanedione in the conjugated form.

Dr. DiVincenzo: Dr. Angelo has studied 2,5-n-hexanedione metabolism and that material was presented here 2 years ago. He found that the hexanedione formed was very extensively converted to CO₂. If I were to speculate on the metabolism of this particular compound I imagine that there would be some initial alpha and omega oxidation. Perhaps both. The alpha oxidation of that compound would give rise to an alpha ketol acid, which in turn would undergo some decarboxylation and perhaps give rise to an acid which could conceivably be conjugated by glucuronic acid. In addition to that, it's feasible that this material can also be hydroxylated and this in turn can give rise to a glucuronide. I don't believe you can form a glucuronide of 2,5-hexanedione without some additional metabolic changes occurring. In our studies we found that a very small amount of methylbutylketone was converted to 2,5-hexanedione and was then eliminated unchanged in that manner. I think it was about 5% of the urinary radioactivity which would calculate as about 2% of the initial 200 mg/kg MBK dose.

Dr. Spencer: Do you want to respond to that, Dr. Abdel-Rahman?

Dr. Abdel-Rahman: The problems start when we speak about 2,5-hexanedione. We used beta-glucuronidase to try to give glucuronic acid. We believed that we should use beta-glucuronidase specifically for glucuronidation. Mass spectra revealed that it was still 2,5-hexanedione in the urine after you incubated with beta-glucuronidase overnight. We got about 20 mg/% excreted in glucuronide form and 1 mg/% excreted as the parent compound.

Dr. DiVincenzo: Dr. Richardson suggested that perhaps there is an enolization taking place and a conjugate may be formed of the enol form and then that conceivably might be cleaved and eliminated in that fashion. I think that is theoretically possible and that would be, I think, the only possible explanation that one could come up with from our understanding of at least the formation of glucuronides. You can't attach a glucuronic acid moiety to a carbonyl as such. Whether or not you are going to have sufficient enolization occurring in a medium such as urine I really don't know. I haven't come across many studies that suggested that's the case.

Dr. Spencer: Dr. DeCaprio, did you have anything to add on that point of discussion?

Dr. DeCaprio: I was going to mention the same thing. Since the reaction between 5-hydroxy, 2-hexanone and 2,5-HD is reversible you could just be seeing the 5-hydroxy,2-hexanone being conjugated and then excreted and then when you conduct the hydrolysis procedure it may revert back to the 2,5-hexanedione species.

Dr. DiVincenzo: It's conceivable that that can take place but not enzymatically. Another point to bear in mind is that there are a lot of contaminants present in most grades of beta-glucuronidase and there could be some materials present that could influence these results and give you a misleading finding.

Dr. Spencer: It's rare to have such a distinguished panel assembled together so I am going to spring something on them and ask for their response. Could we have that slide, please? When we were all patting ourselves on the back for having understood the molecular mechanism underlying hexacarbon neurotoxicity and we were all saying that clearly gamma diketones were the important molecular species in terms of inducing neurotoxicity, there was occurring in 1979 in Texas an unfortunate incident in which individuals involved in the manufacture of plastic bathtubs were using this compound, Lucel-7. These individuals, after only a period of two weeks, developed a flagrant polyneuropathy with some of the other neurological features that we associate with exceptionally severe axonal degeneration. This compound, Lucel-7, has its fifth carbon group blocked by an additional methyl group and so it is 5-methyl, 2-hydroxylated, and I think that we would have predicted that that specific compound would have been without neurotoxic activity. In fact, when we tested 5-methyl,2-hexanone we found that it was free of neurotoxicity. And so I suspect that in 1979, having understood precisely the molecular basis for hexacarbon neurotoxicity we would have had no concern about this compound going onto the market. Yet with this additional 2-azo,terbutyl group on the second carbon we have somehow acquired a neurotoxic potency that is substantially greater than 2,5-hexanedione.

I would like to ask for comments from our distinguished panel and from anyone else in the audience. Is this related to the phenomenon that we have talked about today? Is there an explanation that fits into the very nice hypothesis we have had presented today with regard to gamma diketone neuropathy or is this in fact likely to be an entirely separate mechanism? In the neuropathy caused by Lucel-7 the axons break down in a similar pattern but there is no neurofilament swelling which precedes the degenerative event. This neuropathy looks a little bit like organophosphate poisoning.

Dr. DiVincenzo: We did study 5-methyl, 2-hexanone for its neurotoxic effects and it is relatively nonneurotoxic. We have also studied its metabolism and it poses an extremely complex metabolic picture. I would think that it can conceivably lose that hydrazine moiety and be converted to 2-hydroxy, 5-methyl-hexane but I can't honestly say that I can see a metabolic pathway that would give rise to a potential neurotoxin similar to what we have established, let's say for the hexacarbons.

Dr. DeCaprio: I agree with Dr. DiVincenzo. But I think it would be worthwhile to look at some of the binding characteristics of this compound especially regarding protein binding to see if it does react with either sulfhydryl or amino groups. At least that would provide us with a start.

Dr. Spencer: Does anyone else wish to comment on that particular point or anyone from the audience?

Dr. Richardson: I'm really putting myself on the line since we have people here who should know the answer. Would it be possible to oxidize one of the carbons on the far righthand side of that molecule and then decarboxylate it and be able to produce then a hexacarbon compound which could then undergo further oxidation to form 2,5-hexanedione itself? Then you would have at least a neurotoxic species and you might question the other species that are formed in terms of potentiation of the effect of the 2,5-hexanedione that might be generated. Is it possible to get 2,5-hexanedione out of that?

Dr. DeCaprio: Yes, actually it is. However, I think on a quantitative basis the amount of dione generated would be extremely small. Since this material was apparently more neurotoxic than 2,5-hexanedione, it would be hard for me to conceive of a situation that could generate that much. If you would put up that slide again, please. If you look at the structure of methylisoamylketone and you go over to carbon number 4 next to the branched chain, you can see that it can be hydroxylated with an omega-2 hydroxylation followed by oxidation of the terminal carbon, and that will give rise to a beta-ketoacid that will spontaneously decarboxylate to give the form hydroxymethylbutylketone and then that can undergo an omega-1 oxidation to give rise to the double diol in that case and then oxidize out further to generate hydroxy 2,5-hexanedione. In fact, that happens. But once again, one has to go through a lot of manipulations in order to bring that about. I can't see that as a feasible explanation for the neurotoxicity of the compound in question.

Dr. Spencer: To be fair, I think one has to say that, judging by the outbreak of neuropathy in this Texas plant one would strongly suspect that the neurotoxic potency of this compound greatly exceeded that of n-hexane and probably methyl-n-butylketone. Although there have been no specific studies looking for the comparative neurotoxic potency with something like 2,5-hexanedione, fortunately we have never really seen what happens to a human exposed to 2,5-hexanedione chronically and specifically. So it may be that this compound is no more potent than 2,5 HD itself. Those comparative experiments have yet to be done.

I will terminate this portion of the discussion by making the point that while we can develop these very elegant and very satisfying hypotheses about the mechanism-of-action of hexane derivatives, we must be extraordinarily cautious in making the next step. We must be cautious in saying that we now understand the structure activity relationships between one compound and a second because, as I point out, this particular agent would not have been suspected as a potent neurotoxin. Anyone who tells you that we understand precisely the structure activity relationships of chemicals to distal axonopathies from hydrocarbon compounds will be misleading you at this time.

OPEN FORUM II

Dr. Veronesi (U. S. Environmental Protection Agency): Dr. Sprague, did you find the sciatic nerve degeneration to be symmetrical?

Dr. Sprague (Stauffer Chemical Company): Always.

Dr. Veronesi: And also the CNS?

Dr. Sprague: That's right. As I mentioned, we examined both cross and longitudinal sections of spinal cord and we looked for the specific areas along ascending or descending tracks where lesions would be found. And originally we did not sample bilaterally but we found that there were cases of organophosphate neurotoxicity where we found mild damage and we used the bilateral location to differentiate whether or not it was in fact treatment related. That helped us several times.

Dr. Veronesi: And did you find that spinal cord degeneration preceded the sciatic nerve damage or not? Did you do any kind of a time course study on it?

Dr. Sprague: Yes, we have done some time course work and have also done some reversibility work and the time course as far as we can tell seems to vary often with the compound we use. Sometimes we'll see characteristic lesions in cervical cord and sacrolumbar cord first and with some of the phenylphosphonothioates it's not unusual to see some changes in thoracic cord first. We have not conducted these studies in a systematic way.

Dr. Veronesi: Did CNS damage precede peripheral nerve damage with TOCP?

Dr. Sprague: Yes, usually.

Dr. Van Meter (ICD, Aberdeen Proving Ground): Dr. Sprague, in your description of the various kinds of toxicity, where does chronic toxicity from repeated administrations fit?

Dr. Sprague: I haven't been discussing chronic toxicity today.

Dr. Van Meter: Haven't you entertained the role of the chronic toxicity which develops within a day or two after exposure and the impact that that has on delayed neurotoxicity? Have you looked at that?

Dr. Sprague: No we haven't looked much at chronic long term exposure.

Dr. Richardson (University of Michigan): I think we need a point of clarification on that. Were you talking about the confusion that sometimes arises when you are trying to establish

whether or not you have a clinical effect of delayed neuropathy versus a delayed cholinergic effect especially when you have a compound that may be fairly fat soluble and is being slowly released and causing cholinergic effects? Perhaps over a period of couple of days. Is that what you were getting at?

Dr. Van Meter: I was thinking primarily of those substances which will continue to act like DFP, then be producing a delayed neurotoxicity but in themselves would also produce chronic toxicity. Only I think, because that's the only one that has really been looked at, there may be some others that have an early onset. I'm talking about before you see this profound breakdown of the animal in what you call clinical neurotoxicity. You can see the symptoms, clinically. Something which is occurring at the fine structural level that predicts that something will be occurring within a few weeks. There seems to be confusion on those two issues, mainly in terms of the toxicity which is being developed.

Dr. Richardson: Yes, I think there's been some confusion, because in the early literature you will sometimes see this referred to as chronic demyelinating disease or chronic neuropathy, but the same syndrome results from chronic or acute exposure.

Dr. Sprague: Delayed neurotoxicity from organophosphorus esters can result from chronic administration of these compounds as well. The onset would be slightly different but we would still see the same symmetrical peripheral axonopathy with different onset and it usually occurs at lower doses too.

Dr. Richardson: There may be chronic doses of these compounds that can be tolerated and of course that is one job of the practical toxicologist to find out what that level is.

Dr. Spencer (Albert Einstein College of Medicine): Dr. Jortner, I believe I noted a striking and fascinating discrepancy between the pathology that you described in the spinal cord from paraffin sections with silver stains and the pathology that you described in epon sections and by electron microscopy. What I refer to here is the fact that in the peripheral nervous system examined by electron microscopy you reviewed the accumulation of tubular fascicular profiles and yet you have shown very nicely in the spinal cord tracks that were affected (in the paraffin sections) there was a large accumulation of argyrophilic material which to the ultra-structuralist might appear as an accumulation of neurofilaments. My questions are: 1. Are these accumulations of neurofilaments? 2. Is there any association between dose of the test agent or potency of the test agent and the appearance of these argyrophilic swellings? 3. Do they always appear as a preliminary to nerve fiber breakdown in OP intoxication?

Dr. Jortner (Virginia-Maryland Regional College of Veterinary Medicine): I'm not sure I can answer the question relating to dose relationship. One sees swelling of the central nerve fibers rather readily and can sometimes identify, particularly in the earlier stages, the increased intensity of silver staining. Many years ago John Prineus described the ultrastructure of these changes and did note in addition to some increase in neurofilaments a striking increase in membranous profiles in the swollen central fibers.

Dr. Richardson: Dr. Anderson, do you think that your findings of decreased relative refractory periods are consistent with Lohnes' results on post-tetanic potentiation?

Dr. Anderson (Warner Lambert/Park-Davis): Well, there are two different things going on. With Lohnes' results we are looking at the nerve terminal and there is a loss or change in excitability at the nerve terminal. What I'm suggesting is that when the relative refractory period change starts to occur the nerve axon is compensating in the other way to make sure that behaviorally there is as much normal function as is possible. So things are going in opposite directions to try and maintain homeostasis, if you will.

Dr. Olajos (U.S. Army, ABG, Maryland): Dr. Schwab, my question goes back to the classic studies on cholinesterases. Didn't those studies utilize a relatively purified form of the enzyme?

Dr. Schwab (University of Michigan): Yes, they did and this one did not. And I don't know how you sort that out. Do you know, Dr. Richardson?

Dr. Richardson: Well, it's been done in two ways. It's been done in impure preparations with acetylcholinesterase using recovery of activity as the criterion for aging. Recently Williams and Johnson published studies where they had separated NTE by SDS polyacrylamide gel electrophoresis and showed that the transferred R group was found in the band that corresponded to NTE. It has actually been isolated, at least analytically.

Dr. Olajos: What inroads have been made as to the purification, isolation, and characterization of NTE? I know those of us who have followed the development of this mechanism aspect in chemically induced neuropathies are hoping for major breakthroughs into this area. One of the few studies I am acquainted with that have attempted to address this aspect is the work by Barry Wilson, University of California, Davis. Could you comment as to the inroads that your particular laboratory has made?

Dr. Schwab: As Dr. Richardson said, Williams at least has it on a gel. That also was done in our laboratory by Jane Huggins before I arrived. That is on an SDS gel and it's in a totally inactive form. You have to label the protein in order to find it on your gel. I think Barry Wilson's approach to it has been differential centrifugation over sucrose density gradients and I don't know how successful he has been. Our approach to it has been to try to solubilize the protein and to run it through conventional column chromatography. We have also considered trying to use HPLC to separate the compound. It's from trying to solubilize the protein that I can say that it is intimately membrane bound. It has been extremely difficult to solubilize that protein or at least to solubilize it in an aqueous phase without appreciable loss of esterase activity. We can "solubilize" it in 0.1% triton, where what we have probably done is insert the protein into a detergent micelle. Now that may run through a column but a lot of its sites are being hidden and obviously its molecular weight has been greatly changed by the fact that it is sitting in a micelle. Our approach has been to chromatograph it but it hasn't been enormously successful as yet, primarily because of the difficulty in solubilizing the protein. We can get two or threefold purifications of the protein but we really can't separate it from all the other proteins. We have a long way to go.

Dr. Olajos: The question I have for Dr. Richardson is going back to the trying to pinpoint conceptually the biochemical lesion in OPIDN. An intriguing suggestion was made that the R group is cleaved after the aging of the enzyme and then interacts at a secondary site on NTE. That's very interesting to me. I would like to have Dr. Richardson elaborate a little bit more on that and then perhaps give us his opinion as to the formation of the negative phosphoryl group itself as being deleterious to the neuron.

Dr. Richardson: I think Dr. Schwab alluded to some of the possibilities for the negatively charged phosphoryl moiety being deleterious to the axonal membrane. One hypothesis that has been around for awhile is one that M.K. Johnson proposed that what we are doing is providing a spurious phosphoprotein signal which mimics the phosphoprotein signal that would be generated by endogenous protein kinases depositing their gamma phosphate onto a receptor protein, thereby entering into some sort of regulation of cellular activities. Those are now known to be commonplace and widespread throughout neurons. We suggested some years back that NTE may be a receptor protein for a protein kinase and it may act in an endogenous protein kinase system. We have now accumulated some evidence, but not enough, to totally rule out that hypothesis but, enough to say that that looks now even more unlikely than some people told me it was in the beginning. We are left with the idea that it still could be quite likely that we have created a spurious signal. The biological precedent for this is the activation of oncogenes leading to protein kinases which in turn lead to either abnormal phosphorylated proteins or

abnormally high levels of phosphorylated proteins which disrupt cellular metabolism or cell membranes in some way. In the case of oncogenes you have a cell that becomes malignantly transformed. In the case of our hypothesis, you would have an axon membrane that can no longer cope and it begins undergoing degeneration. Now this is wild speculation at this point but it is something we offer as a possibility. The other way we can wave our hands about a negative charge is that we must be accumulating negative charges in the membrane and there has to be some compensatory mechanism for balancing that excess negative charge. It may tie into what Dr. Anderson has observed that there are electrophysiologic shifts which indicate that there is a compensatory mechanism. The shift of the R group to what Johnson calls site Z is a little harder to believe, initially at least, as something that is actually involved in pathogenesis. It seemed very tidy when you are thinking of generating a negative charge because you are always generating the same species no matter what the compound is. There are a wide variety of organophosphorus structures that when they do inhibit NTE will lose that R group and result in a negative charge even though there is a wide variety in the types of structures that leave in this aging reaction. It seemed far-fetched to think that this wide variety of R groups could all be deposited somewhere and lead to the same kind of defect. But now we know that the transfer for a wide variety of R groups seems to be stoichiometric. It occurs with 100% efficiency. It seems to be a SN-2 attachment reaction rather than a SN-1 type of reaction which seems to be favored in aging of cholinesterase.

We haven't said much about nerve agents in this series of talks and I know that there are many people here with an active interest in nerve agents. It turns out that the rate of aging of the nerve agent Soman, the loss of the pinacolyl group on NTE has a half-life of about 10 hours. Whereas on cholinesterase the same group leaves and it has a half-life of about one minute. With the other compounds where a SN-2 type of reaction would be favored, the neurotoxic esterase will age at very rapid rates. Usually less than 5 minutes for a $T_{1/2}$. We think that the biological precedent for a R group interaction might be something like interference with regulation of receptor sites by methylation processes. We know that occurs in a wide variety of receptors. The one that has been best characterized recently is the aspartate and serine receptor from bacteria. There are mammalian receptors that also are known now to be regulated in part by methylation and so if you were transferring this R group to a site that normally is methylated, it's conceivable that that could be the perturbing event.

Dr. Klemme (NIOSH): I am not familiar with this field of research but, from some of the data presented today, I was caused to wonder about the possibility of an immunological process going on with the alkylated NTE and in particular whether the alkyl group is either a hapten or is causing a conformational change of another exposed part of the protein. This type of process would

be suggested by the one week gap in the effect and the one to four week onset of maximal clinical effect and its persistence for up to a year. I don't remember whose data it was but someone showed for one compound that two separate doses of 500 mg caused a greater effect than a single 1500 mg dose of an organophosphate. It might also be consistent with plasma bound protein and with protection of non-hapten producing substrate.

Dr. Richardson: Yes, I happen to think that is an excellent suggestion and it is something I have thought of also. I have had a hard time selling it to people. When I talk to immunologists about this they are very skeptical and they point out that you don't see the normal sort of inflammatory response that they often associate with an immune mediated process. Maybe some of the neuroimmunologists in the audience could comment on that. It seems to me since we know so little about immunology and its changing so rapidly every day that there might be possibilities for that kind of a mechanism. There is some negative evidence that has been provided by Sharma and McNulty at Utah State where they did some preliminary experiments in trying to ablate the immune response by removing organs having to do with mounting an immune response or radiating the animals. They found that the response to TOCP was not changed at maximal doses. I think that the experiments need to be looked at with greater refinements in order to come to a definitive answer about whether there is some sort of autoimmune mechanism. One intriguing thing that recently has come out of our lab is that NTE is found not only in the nervous system but also in the lymphatic system. We find a great abundance in the spleen of the chicken and in circulating lymphocytes. It seems to be found in both T and B cells. We haven't done very exacting studies as yet to track this down. The way we have been pursuing it in my lab is that we are taking a practical attack and using lymphocyte NTE as a biomonitor of exposure to organophosphorus compounds that might be neuropathic. We are also interested in these basic questions about whether or not there may be an immune mechanism. I think it's a well considered suggestion.

Dr. Schwab: You should add to that information the fact that the group at Cornell has shown that one can protect against organophosphorus neuropathy with massive doses of corticosteroids given at the same time as the toxic organophosphate. That study was a little bit crude in that one can't really tell what you have done to absorption and distribution and the compounds by giving massive doses, and they were truly heroic doses, of organophosphates. No explanation was offered in the literature but it's an interesting thought.

Dr. Jortner: Dr. Marian Erich, who is an associate of mine, has had mixed results with corticosterones. She and Dr. Gross recently published a paper showing a protective effect against TOCP induced delayed neuropathy in hens and there is a paper that

has been submitted for publication from her group that shows no corticosteroid protective effect with DFP induced toxicity. I think the jury is still out on that issue.

Dr. Abdel-Rahman (New Jersey Medical School): The toxic response to organophosphate declined very rapidly after multiple exposure. What sort of adaption to the organophosphate occurred? When you give malathion or any organophosphate by injection, the first day of the experiment you have a very high toxic response. A second injection given after 24 hours gets a toxic response that declines very rapidly and it appears that some sort of adaptation occurred.

Dr. Richardson: I think Dr. Schwab would be the best one to comment on that.

Dr. Schwab: I spent a lot of time looking at that response prior to my interest in organophosphate neuropathy. That tolerance phenomenon is one which occurs with an awful lot of anti-cholinesterase compounds such as carbamates and organophosphates and you can probably induce it with direct cholinergic agonists as well. You can induce tolerance to things like carbamyl choline. The work that I did in that area suggested that the loss of toxic response to an organophosphate was attended by a decrease in muscarinic-cholinergic receptors so that what the system was doing was actually shutting down its sensitivity to the end product of anticholinesterase type of organophosphates. It was no longer responding to acetylcholine because it didn't have the receptors to do it anymore. This response wasn't directly related to organophosphate structure at all, and one could induce it with almost any inhibitor of acetylcholinesterase or even with direct agonists. That's in contrast to the fact that one can produce an organophosphorus neuropathy in animals treated for long periods of time with an organophosphate with just the appropriate dose to inhibit an appropriate proportion of neurotoxic esterase. And that proportion of neurotoxic esterase doesn't really change whether you use an acute dose of the compound or whether you give many doses and ultimately reach that amount of phosphorylation of NTE. The organophosphate effect that you are talking about is probably an effect of an anti-cholinesterase compound and no such tolerance exists as far as neurotoxic and delayed neurotoxic response to organophosphates.

Dr. Richardson: Let's be very clear on distinguishing the two effects. The kind of tolerance Brad is talking about is that which can be produced by cholinergic agonists or by compounds that produce an increase in acetylcholine concentration. We're talking about tolerance to the acute effects or the anti-cholinesterase effects of compounds. With regards to the other comment he made about the threshold of NTE inhibition being required for neuropathy, with a single dose the threshold is about 70% inhibition but we think now that it is probably improper to talk about the level of inhibition in terms of the threshold especially when you get into chronic toxicity. Because actually there is a bit

of a change. There are four or five studies published in the literature now showing that while they disagree on the level of the threshold of inhibition, they do indicate that the level of threshold for chronic exposure to produce neuropathy is a bit lower than it is for a single shot. Whereas it may be 70% for a single shot of TOCP in a hen, chronic dosing with TOCP may produce the effect with as little as 55% inhibition of NTE. Some people say a little lower, some people say a little higher. What probably has to be considered here is not the precise level of inhibition of this enzyme, because the enzymic activity doesn't seem to matter anyway, but the level of altered protein. How many of these phosphorylated sites have you produced? Right now we don't have a convenient way of counting those, but I think if we did we would find that there may be some constant number. What we need to be looking at is the rate of decline of these phosphorylated proteins once they are produced.

Mr. Vernot (University of California, Irvine): My question is for Dr. Schwab, although another speaker also showed the same slide which had to do with the kinds of phosphorus esters which produced delayed neurotoxicity and the kinds that didn't. I was confused because what the slide called a phosphate I would have called a phosphonate. There were two ester groups on the molecule. What it called a phosphonate I would have called a phosphinate. I have seen the same chart in a text and it confused me there as well.

Dr. Schwab: I have a little trouble with phosphate nomenclature myself and I generally follow the nomenclature that Ito uses in his book. Wherein a phosphate would have three phosphorus oxygen bonds and then the double bond to the oxon portion. A phosphonate would have a single ROP bond and then there would be a phosphorus carbon bond in lieu of that second ester. A phosphinate would only have an ester bond to the leaving group, the group that would leave when it phosphorylates protein.

Dr. Richardson: Yes, I think it becomes clear when you just think of a phosphonate as a derivative of phosphonic acid and of phosphinate as a derivative of phosphinic acid and so on. And where the confusion may come is if you draw a triester you would have, say a triester phosphate and then you have three ROP bonds to the P double bond O. If you indicate one of those as a leaving group by an X, where you have the fluorine for example, then you are talking about the corresponding acid fluoride and what you really need to do is put in the other triester bond and I think that would clear it up. Does that make it any less muddy?

Mr. Vernot: I think so. I do have another question for Dr. Anderson and I was wondering when she was talking about her electrophysiological measurements on the rat which is a resistant species to delayed neurotoxicity, whether she had considered doing similar work using a sensitive species such as the hen.

Dr. Anderson: Yes, that is in our plans, and as a matter of fact I am collaborating with Dr. Richardson now who has a flock of hens and that's one of the things we have discussed.

Dr. Yang (NEIHS, NTP): I need a clarification from Dr. Schwab. On your last slide you showed a flow of events of OPIDN and between the bouts of injury and repair there are two arrows going one way. Shouldn't one of the arrows go the other way around?

Dr. Schwab: I would have to look at the slide to tell you whether it's just a typo or whether there was a reason for that. That's Dr. Richardson's slide, I might add.

Dr. Richardson: Yes, I guess I am the culprit there. I drew that. I had intended to show that one was a dash arrow with a negative sign and the other was an arrow with a positive sign.

Dr. Yang: But those are going from injury to repair as I see it. Shouldn't one be the other way around?

Dr. Richardson: The way we had conceived this model was we were trying to incorporate the possibility for regeneration and repair to explain the anomaly of age and species differences in sensitivity. Given that different ages of animals and different species of animals will have different capacities for regeneration and repair in their nervous systems when they are subjected to some kind of injury. What I was trying to say is that the existence of injury in this cell would act as a trigger to stimulate repair processes. That would be the arrow going up with the positive sign over it. If you have overwhelming injury accumulating at a rate faster than repair can take care of, then you also compromise repair processes and that's the arrow with the negative going up. Meaning it's a negative impact on repair.

Dr. Yang: Thank you.

Dr. Spencer: Dr. Schwab, is the target protein likely to be serine rich or not?

Dr. Schwab: Dogma would say that if you are phosphorylating with something like DFP probably the site phosphorylated is a serine hydroxyl group. Whether or not that means the entire protein is serine rich or not I don't know but I don't think so. That hasn't been unequivocally demonstrated, however, and it's something we want to do. It is perfectly conceivable that

the site of phosphorylation is a tyrosine hydroxyl group. It's interesting to note in light of what Rudy talked about in terms of oncogene products, that things which are phosphorylated by kinases that are stimulated by oncogenes are very often phosphorylated on tyrosine residues as opposed to serine enzymes. That may make the investigation of what that phosphorylation is perhaps even more interesting. If somebody put a gun to my head I think I'd have to say it's probably a serine hydroxyl site that is phosphorylated.

Dr. Spencer: And to what does the leaving group attach and how close is that to the serine site?

Dr. Schwab: That's another thing that we don't know. The thing that interests me in that area is whether all these different R groups which are leaving are in fact attached to the same site. Even that hasn't been demonstrated. I would suspect that they are but we don't know what that site is.

Dr. Schwab: I would like to answer a couple of questions that Dr. Griffin posed in his review.

Dr. Richardson: Okay.

Dr. Schwab: We have a grant to look at the distribution of NTE right now with Matt Miller and Peter Spencer's group. You also asked about the axonal transport of NTE and we have a grant to look at that, but we may have been scooped. At the neuroscience meetings last week a member of Dr. Abadonia's group apparently reported on the axonal transport of NTE but I haven't even had an opportunity to read the abstract so I don't know what the findings were. Our preliminary findings were that NTE is transported in the fast axonal transport component but that's very crude. Then there was one more experiment that you asked about that actually Dr. Richardson, Dr. Spencer and I did together and that was to look at whether NTE is a component of axonal membranes or whether when one grinds up a nerve or a brain or a spinal cord and looks at this membrane-bound protein whether maybe it's not in some sort of a glial fraction. We knew from earlier work of Dr. Richardson's that it wasn't in myelin but we didn't know whether it was in glial plasma membranes. To that end we denervated some cats and waited for five weeks and in that preparation we found that there was still neurotoxic esterase. We only got to do it once because we ran out of money but it looks like up to 50% of NTE activity in a preparation is actually in Schwann cells. That's an experiment that bears repeating though.

Dr. Hansen (University of Illinois): I remembered what the lines were. I was interested in your duration and intensity of inhibition of NTE. We found the same sort of thing comparing leptophos or ethoxy leptophos in the hen. Our problem was we ran out of money and compound so we didn't get it refined enough to publish but we found 75 to 85% NTE inhibition with the ethoxy

leptophos at about 1/4 the effective dose and 85 to 95% inhibition with leptophos at the same dose level. This was at 24 hours posttreatment. The leptophos inhibition reached this high level much more rapidly and maintained it for a longer time than the ethoxy leptophos. It went up to maybe 85% at 24 hours and by 48 hours was down again to 72%. We shifted then because we were running out of pure compound. We were using analytical standard and oral doses of 300-350 mg/kg don't last very long. So we went to intravenous doses and we knew what the effective IV dose was, 35 to 40 mg/kg for leptophos, but even at a 25 mg/kg dose we had 100% NTE inhibition at 24 hours but by 72 hours it was recovering considerably.

Dr. Richardson: I think you hit on the point that maybe wasn't stressed. That when you are using this as a test to ask the question will a compound produce delayed neuropathy in a single dose situation. If the compound is capable of producing in a single dose a supra-threshold inhibition which we think is about 70% in whole brain, 24 hours after dosing a chicken by whatever route you can expect that neuropathy will follow in a pair-dosed bird. There is sometimes the misconception that the NTE level will be depressed most near the time when they would expect neuropathy to be present. Say 14 days. They assay for NTE and they find that it's maybe at control levels and they are puzzled by this. The correct spatial temporal organization has to be to regard a high level of inhibition as an initiating event. So what you want to do is look at the level at time 0 or 24 hours after dosing and that level will correspond to whether or not you have neuropathy at 14 or 15 days after treatment. The same thing occurs with chronic dosing. Once you achieve whatever the threshold is for the chronic schedule you are using which may be 50% NTE inhibition then 14 days from that point you would get neuropathy. We know that NTE comes back almost totally by resynthesis rather than regeneration of enzyme and the half-life for resynthesis has been measured by Johnson and by Corauldi and Lotte and they come up with comparable figures where half-life is around about 4 to 7 days either in spinal cord, brain or peripheral nerve and so it's comparable to a number of other proteins that are turning over and being resynthesized.

Dr. Hansen: I haven't worked as much with NTE as you have but in the intravenous dosing is it possible that we trap a lot of leptophos in the nervous tissue and then it's released when we homogenize the tissue and in this case inhibits more NTE than really is inhibited in the in vivo situation?

Dr. Richardson: I guess it's possible.

Dr. Hansen: We kept having to lower the dose down to get below 100% inhibition at 24 hours.

Dr. Richardson: Oh, I see, you are actually getting the number that if you just did the sum you would get something greater than 100% inhibition. That I can't explain because in a

differential assay if you were biting into the CIRE activity it would cancel between the two tubes. So something peculiar is going on. Leptophos and its derivatives have been puzzlers.

Dr. Hansen: There is one other thing that we had to do that I didn't mention that maybe has some bearing. We had to give the I.V. dose in about 25% ethanol. The final formulated dose. The chickens do stagger for three to five minutes after they receive this intravenous dose. Would that have any effect on the NTE?

Dr. Richardson: That might. In in vitro studies we find that the presence of organic solvents sometimes actually stimulates NTE activity. We sometimes do in vitro studies with inhibitors where we dissolve the inhibitors in acetone and have a final acetone concentration of about 3% in an aqueous medium. And we find some stimulation of NTE so that might be a possibility.

SESSION III

TOXICITY TO THE MALE REPRODUCTIVE SYSTEM

Chairman

**James W. Overstreet, M.D., Ph.D.
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**OCCUPATIONAL EXPOSURES AND REPRODUCTION:
SITE AND MECHANISM OF ADVERSE EFFECTS**

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INTRODUCTION

In addition to potential adverse effects on health, working may impact on the fertility of men or women, or alter the course of pregnancy. Adverse effects on reproduction may occur either as a result of exposure to a toxic chemical or the effect of physical exertion on the reproductive processes. Because reproduction is such a crucial process for humankind in general, and for the individual in particular, and because work is essential, it is important that we develop a comprehensive understanding of the effects of work on reproduction. Characterization of the effects of work either inside or outside of the home on fertility and reproductive performance is essential because just as there are occupations or exposures which may impair reproduction, there will also be occupations and exposures which have no effect on reproductive function.

Reproductive toxicology is a multifaceted discipline - we encounter this complexity most clearly when we try to define the effects of occupation on reproductive function. One of the complicating factors of this topic arises from the unique nature of reproduction. Reproduction function is not like renal, pulmonary or hepatic functions in several ways: 1) reproduction requires two individuals, clearly not the case for other organ systems, and 2) reproductive function is a central part of the personality of the individual, complicating assessment of reproductive function in an occupational setting. For example, in an environment where masculinity is equated with an active sex drive, exposures which alter libido or sexual performance may be denied by male workers. These factors may make the assessment of reproductive function difficult in many occupational settings. However, it is important not to be discouraged by these difficulties but to begin to develop methods capable of assessing interactions between occupation and reproduction.

Because reproductive toxicology is such a complex science spanning many disciplines, I will begin by defining successful reproduction, and then use that to define what is meant when we say that a drug, occupational or environmental exposure is toxic to reproduction. Following definition of a reproductive hazard I will review a medical history form which was developed by the American College of Obstetrics and Gynecology which includes suggestions for obtaining occupational data. This is important because one major deficit in reproductive toxicology is the provision of adequate data. Then I will review the effects of selected occupational exposures on reproduction.

CHARACTERISTICS OF REPRODUCTION

Reproductive function must be evaluated differently from the function of other systems in the body. In addition to requiring two individuals for successful function, reproductive function is not expressed continuously. In humans and animals a variety of hormonal controls exist which regulate the expression of reproduction (Takizawa and Mattison, 1983). For example, fertility among wild animals falls during times of stress or nutritional deprivation, and similar decreases in fertility may also occur in humans (Cumming and Rebar, 1983).

The reason for the high degree of regulation of the reproductive system is the cost of reproduction. In humans and animals pregnancy imposes a metabolic burden on the maternal organism. In general, the maternal organism is able to adapt and successfully complete the pregnancy (Hytten and Leitch, 1971). However, in some cases prior exposure or exposure during pregnancy can impair the outcome. Later I will discuss a unique form of occupational hazard to reproduction, pre-pregnancy exposure to beryllium alters pulmonary function and impairs the maternal pulmonary adaptation to pregnancy. Women with beryllium lung disease have a significantly increased risk of death during pregnancy (Barlow and Sullivan, 1982).

Because of the metabolic and economic costs of reproduction, we have developed a variety of techniques for controlling the expression of fertility - most notably, oral contraceptives. This is also unique - most medications are given as replacement for failed organ systems, or to control the responses of organ systems which have lost control. In contrast, oral contraceptives are given to subvert the normal integrated control of the reproductive system.

SUCCESSFUL REPRODUCTION

These considerations suggest that successful reproduction has two components: 1) the production of healthy offspring, when fertility is desired, without excessive risk to the maternal organism, and 2) safe and effective contraception when fertility

is not desired. Successful reproduction is therefore the ability of a couple to produce healthy offspring safely at the appropriate time in their life cycle.

REPRODUCTIVE TOXIN

By this definition a reproductive hazard is anything which impairs fertility when it is desired, increases the production of abnormal offspring, or decreases the effectiveness of a contraceptive. Before we begin to review occupational exposures which impair fertility, I want to expand on the concept of a reproductive hazard acting to decrease the effectiveness of oral contraceptives. Over the past several years and especially with the introduction of the low dose oral contraceptives, physicians have noted contraceptive failures in association with certain other medications (Breckenridge et al., 1979). Oral contraceptives containing an estrogen and progestagen act by providing continuous low level estrogen feedback to the hypothalamus. This exogenous estrogen acts to block the ovulatory gonadotropin surge. If a drug or occupational exposure increases the rate at which these exogenously administered steroids are cleared from the blood, feedback on the hypothalamus and contraceptive effectiveness will be diminished.

The two major classes of drugs implicated in decreasing contraceptive effectiveness are antibiotics and anticonvulsants, drugs which influence hepatic metabolism or enterohepatic circulation of oral contraceptives. It is important to note, however, that not all antibiotics or anticonvulsants have this adverse effect.

Most surveys of occupation and fertility do not address this issue of contraceptive effectiveness. In fact, these studies are designed to measure decreases rather than increases in fertility. However, one study conducted several years ago in the United States did observe an increase in fertility for workers in one plant. Unfortunately, analysis of the data fails to reveal if this is a result of contraceptive failure, or simply an economic effect, with pregnancy reflecting the economic security of the job (Levine et al., 1980). Additionally, one study of the wives of men exposed to chlorinated dibenzodioxins suggested an increased contraceptive failure rate (Towsend et al., 1982).

OCCUPATIONAL WORK HISTORY

One of the biggest problems facing reproductive toxicology today is one which we can all work together to solve. This problem is a lack of data which can be used to evaluate the effect of occupation on reproduction. Several years ago the American College of Obstetrics and Gynecology recognized that this was a major deficiency. The history form subsequently developed represents an attempt by the College to provide assistance in gathering relevant occupational information for identification of

occupational hazards to reproduction (The American College of Obstetricians and Gynecologists, 1978). Although the form was designed for women it is easy to adapt for male workers.

The first question explores the type and place of work, and includes information on job title, employer, union, supervisor, and occupational health staff. These data are useful if additional information is needed to clarify the actual exposures received by the couple. Note that information is requested on both the patient and his or her partner. This is necessary because it has been frequently observed that toxic exposures can be carried from a workplace to home on clothes. These "carry home" exposures can act alone or in concert with the work exposure of the partner to impair reproductive function (Hemminki et al., 1983).

The second question gathers information on work schedule, how many days worked per week, and how many hours worked per day. In addition, it is useful to know the flow of work and the frequency and duration of rest periods and breaks. Although severe physical exertion may impair fertility in women (Cumming et al., 1983), the effect on fertility in men has not been explored. The major reason for this question is to alert the health practitioner to work situations which may require adaptation for fertility or pregnancy. For example, if scheduled rest periods are replaced by rest periods which are taken as needed it may be possible for the pregnant women to work further into the pregnancy. This is important because since many women work from economic necessity, cessation of income early in pregnancy may actually be more harmful to the woman and fetus than working into the third trimester.

The third question concerns amenities available on the job site. Are bathroom and rest areas available in convenient locations? Is food, water, or other refreshment available for the worker? Finally, is there quick access to emergency medical care? During the first trimester, when nausea and vomiting are common, and later in pregnancy when the uterus presses on the bladder stimulating frequent urination, convenient access to bathroom facilities is a necessity for the pregnant worker. In addition, the availability of rest periods is meaningless unless there is a place where the pregnant worker can lie down and elevate her legs. Finally, during pregnancy when multiple small meals may be more palatable to the pregnant worker than one or two large meals, the ability to have a snack while resting is important. It is important to note that these facilities do not have to be opulent, they just need to be available.

The fourth question relates to the physical work done on the job - what is actually done by the worker, and whether it is done sitting or standing. What are the characteristics of the physical activities - bending, walking, climbing, twisting - done on the job? If, for example, the job involves standing for long periods of time, this will have an obvious effect on blood return

from the legs as pregnancy progresses. It might be useful to inquire if the job can be done while sitting on a high stool; this would help to decrease venous pooling and fatigue in the pregnant worker.

It is also important to determine the characteristics of the tasks performed by the pregnant worker. What is the weight of carried materials? Does the job require balance, coordination, or frequent rapid movements? Alterations in body shape and center of gravity may make lifting and carrying more difficult for the pregnant worker. These same alterations will also alter her balance and coordination, and make it more difficult for her to move rapidly.

The final question explores the environmental characteristics of the workplace. What is the temperature and humidity, and is there exposure to noise or vibration? Does the job involve exposure to biological agents? For example, bacteria, or viruses. If there is occupational exposure to biologicals? Are these agents infectious or toxic to the fetus? If so, exposure should be curtailed during pregnancy. This also raises an interesting question. Suppose, for example, the husband is a virologist. What steps should he take to prevent carry-home exposure of his wife, or should he even stop working? It is also important to characterize the chemical exposures in the workplace; however, I will concentrate on that topic later.

These five questions will provide occupational health professionals with basic information concerning the nature of the work, and types of exposures in the workplace of a couple. Some of this information is useful immediately in guiding patients away from occupational hazards to reproduction. In addition, collection of these data will also be useful in defining additional hazards to reproduction, as well as defining exposures which have no adverse effect on reproductive function.

EFFECT OF OCCUPATION ON REPRODUCTION

One of the useful tools generated by the Library of Medicine recently was a computer search of the world medical literature on the topic of occupational exposures and reproduction. During the period from 1963 to 1981, there were approximately 270 articles published which explored the effect of work on reproductive function (Pruett and Winslow, 1963-1981a, 1982b).

EFFECTS ON MALE REPRODUCTION

Most of the effects on male reproductive function focus on alterations in spermatogenesis, fertility, testicular function, or hormone production. It is interesting to note that adverse effects on the scrotum are also commonly reported. This serves to remind us that the first occupational cancer identified was

cancer of the scrotum in chimney sweeps in England more than 100 years ago. Later I will review the effects of occupational exposure to dibromochloropropane, a testicular toxin.

FEMALE REPRODUCTIVE EFFECTS

About half of the reports focused on workplace hazards to the pregnant worker, exploring effects on miscarriage and pregnancy. The next largest group of reports - looking at menstruation, fertility, ovary and hormones as reproductive endpoints - investigated occupational effects on the integrated function of the hypothalamic-pituitary-ovarian axis. A smaller number of reports explored the effects of workplace exposures on the uterus, vagina and on libido.

LIST OF REPRODUCTIVE TOXINS

Table 1 lists the chemicals implicated as reproductive toxins by this computer-generated bibliography. First, it is important to state that many of these compounds may not be reproductive toxins. Most of the reports in this bibliography have not been confirmed by other investigators. In addition, the articles collected in this bibliography were not critically reviewed by the authors of the bibliography. With these caveats in mind, however, we can identify compounds on this list that are indeed known reproductive toxins.

Some of the reported reproductive toxicity is trivial - for example, the antibiotic ampicillin is listed as a reproductive toxin because women working in pharmaceutical laboratories formulating the drug report a higher frequency of vaginitis than women not exposed. It is easy to understand the mechanism of this effect - altered vaginal flora. It is also important to recall that just as ampicillin can alter the vaginal flora it can also alter microbial populations in the intestine and may decrease enterohepatic circulation (and effectiveness) of oral contraceptives.

Other compounds listed as reproductive toxins are the synthetic steroids, mestranol and norethindrone. Occupational exposure to these compounds also occurs in the pharmaceutical industry. Table 2 summarizes the effects of occupational exposure in a pharmaceutical laboratory in the United States to norethindrone, a synthetic estrogen used on oral contraceptives (Harrington et al., 1978). None of the 5 women on the office staff, who were not exposed to dust containing the steroids, had intermenstrual bleeding. There were 18 women involved in the production of an oral contraceptive containing norethindrones; 10 of these women had intermenstrual bleeding. In addition, gynecomastia was common among the men working in the production area of the laboratory.

**TABLE 1. ENVIRONMENTAL AND OCCUPATIONAL COMPOUNDS
REPORTED TO ALTER REPRODUCTION**

Acetone	Acrilonitrile	Aldrin
Alkylmercurials	Amitrole	Ampicillin
Aniline	Antimonite	Arsenic
Asbestos	Benzene	Bromoethane
Butanol	Cadmium	Baprolactam
Carbaryl	Carbon disulfide	Chlorobenzene
Carbon tetrachloride	Chlorine	Chlordane
Chlordecone	Coal Tar	Chromium
Chlorinated naphthalines	DDD	Chloroprene
Coal	Diaminoanisole	Copper
Cyclohexanone	Dimethylformamide	DDE
DDT	Dinitrocresol	Dichloroethane
Dibromochloropropane	Enflurane	Dichloromethane
Dichlorophenoxyacetic acid	Ethylene dibromide	Dichlorvos
Dieldrin	Ethyleneimine	Dinil
Dinitrobutylphenol	Formaldehyde	Dinitrotoluene
Dioxins	Glycerine	Ethylene
Ethyl Cellosolve	Hexachlorobenzene	Ethylene oxide
Ethylenediamine	Hydrochloric acid	Fluorine
Fluroxene	Khometsin	Gasoline
Furfuryl Alcohol	Malathione	Granusan
Halothane	Mestranol	Iron
Hexachlorocyclohexane	Methylacrylate	Lead
Hydrogen sulfide	Mirex	Manganese
Lindane	Norethindrone	Methoxychlor
Mercury	Phenoxyacetic acid	Mineral oil
Methoxyflurane	Phosphorus	Nitron
Methylmercaptophos	Polyamide	Oryzalin
Nitrous oxide	Polyhalogenated	Phenthiuram
Phenol	dibensofurans	Phthalates
Phenylenediamine	Polychloropiene	Polystyrene
Plutonium	Hydrocarbons	Pibenzofurans
Polyhalogenated biphenyls	Radium	Polyvinylchloride
Polychloronaphthalene	Silicon	Rayon
polychloropinene	Sodium hydroxide	Silver cyanide
Polycyclic aromatic	Sulfur dioxide	Solder
hydrocarbons	Tetrachloroazoxybenzenes	Synthetic alcohol
Potassium cyanide	Tetrachloroethylene	Tin
Selenium	Toluenediamine	Toxaphene
Silvex	Trichlorophenoxyacetic	Trinitrotoluene
Styrene	acid	Xanthates
Tetrachloroazobenzenes	Tricresyl phosphate	
Tetrachlorodibenzodioxane	Vinyl chloride	
Tetramethylthiuram		
disulfide		
Toluene		
Trichloroethylene		
Tricresol		
Uranium		
Zineb		

TABLE 2. REPRODUCTIVE EFFECTS IN WOMEN OCCUPATIONALLY EXPOSED TO NORETHINDRONE

<u>Job</u>	<u>Number</u>	<u>Number with Intermenstrual Bleeding</u>
Processing	1	1
Quality assurance	5	1
Production	18	10
Office Staff (no exposure)	5	0

From Harrington et al., 1978.

Women working in quality assurance and processing - both areas with smaller exposure - were less likely to experience intermenstrual bleeding, and men in these areas were also less likely to complain of gynecomastia. It is also easy to understand the site and mechanism of action of the exposure - the exogenous synthetic estrogen acts to interrupt the normal endocrine homeostasis and also stimulates cells containing estrogen receptors.

EFFECT OF WORK STATUS ON SPONTANEOUS ABORTION

The results from a study of the effect of occupation on spontaneous abortions conducted in Finland by Hemminiki (Torkelson et al., 1961) are fascinating, and suggest an effect of exertion on reproductive outcome. In preparing their data these epidemiologists initially grouped the women into two categories: those working outside the home, and those women who did not work outside their home. It is interesting to observe that the rate of spontaneous abortions is almost twice as high among those women working outside the home (11 per 100 births) as those not working outside the home (6 per 100 births). At the present time it is not known if this observation can be confirmed, but it suggests that future investigations of the effect of occupation on the frequency of spontaneous abortion will need to include working controls in addition to non-working controls.

SPONTANEOUS ABORTION IN WOMEN IN THE CHEMICAL INDUSTRY

Table 3 summarizes the results of several epidemiological surveys conducted to explore the relationship between occupation and frequency of spontaneous abortion (Barlow and Sullivan, 1982 and Hemminki et al., 1980). In Finland, certain occupations carry an increased risk of spontaneous abortion in comparison with the overall population. These occupations include some sectors of the chemical and metal industries, as well as a broad spectrum of other occupations. At the present time it is thought

that the increases in frequency of spontaneous abortions observed in these workplaces reflects exposure of the worker to some chemical reproductive hazard.

**TABLE 3. SPONTANEOUS ABORTIONS IN WOMEN
EMPLOYED IN CHEMICAL INDUSTRIES**

<u>Employment Status</u>	<u>Number of Spontaneous Abortions</u>	<u>Number Per 100 Pregnancies</u>	<u>Number Per 100 Births</u>
All Finnish Women (1973-1976)	15,482	5.52	7.98
Chemical Workers	52	8.54*	15.57**
Plastics	21	8.94*	17.80***
Styrene	6	15.00**	31.59***
Viscose Rayon	9	11.25*	22.50***
Dry Cleaning	7	10.14	16.67*
Pharmaceuticals	5	10.20	22.72*

*p < 0.05, **p < 0.01, ***p < 0.001.
From Hemminki et al. (1980).

During the 3 year period surveyed there were 5.5 spontaneous abortions per 100 pregnancies, and 8 per 100 births in all Finnish women. Among those women working in the chemical industry there were 8.5 spontaneous abortions per 100 pregnancies, and 15.6 per 100 births - both significantly larger than the overall population. Those occupations with the highest risk of spontaneous abortion were the plastics, styrene, and viscose rayon industries with rates per birth 3 to 4 times higher than the rates observed in all Finnish women. This suggests that some chemical exposure in these workplaces is harmful to the fetus or disrupts the endocrine homeostasis necessary for successful pregnancy.

OCCUPATIONAL EXPOSURE TO ANESTHETIC GASSES

Unfortunately, the health professions are not protected from reproductive toxicity. More than 10 years ago Russian epidemiologists observed an increase in the frequency of spontaneous abortions among anesthetists (Hemminki et al., 1983). A subsequent study of dental assistants demonstrated a dose dependent effect of anesthetic gas exposure on the frequency of spontaneous abortion. Among women with no exposure to anesthetic gasses during pregnancy the spontaneous abortion rate was 8.1 per 100 pregnancies. Those women with light exposure to anesthetic gasses had an increase to 14.2 and those with heavy exposure were further increased to 19.1 spontaneous abortions per 100 pregnancies. More recent observations also suggest that exposure to ethylene oxide, used as a sterilizer in many hospitals and laboratories, also increases the frequency of spontaneous abortion (Hemminki et al., 1983).

In addition to increasing the frequency of spontaneous abortions, anesthetic gas exposure during pregnancy also appears to decrease birthweight. Infant weight of those born to non-physicians in this study was 3430 gms, while for infants born to anesthesiologists birth weight was 3347 gms (Pharoah et al., 1977). Although this difference appears small - the telling statistic is the percent of infants weighing less than 2500 gms - among non-physicians 3.7% of the infants weighed less than 2500 gms while among anesthesiologists 6.2% of the infants weighed less than 2500 gms at birth. This suggests that factors in the workplace environment of anesthesiologists are indeed toxic to reproduction, increasing the frequency of spontaneous abortion, and decreasing birthweight.

BERYLLIUM

Beryllium is an element which is used in the metal industry to produce light, strong alloys. Women exposed to beryllium dust, from carry-home, neighborhood, or industrial exposure, may develop beryllium lung disease. Of special interest to obstetricians are the observations suggesting that pregnancy enhances beryllium toxicity from prepregnancy exposure. That is, women with chronic beryllium poisoning experience a high mortality during pregnancy.

Table 4 lists the precipitating factor or proximate cause of death in a group of individuals who died from beryllium poisoning (Barlow and Sullivan, 1982). Among these individuals the most common precipitating factor was thought to be pregnancy. Additionally, although half of the individuals with chronic beryllium pulmonary disease are women, more than half of those dying with the disease are women. It is not known if this excess is due to pregnancy or other factors which increase toxicity in women.

**TABLE 4. POSSIBLE PRECIPITATING FACTORS
IN FATAL BERYLLIUM LUNG DISEASE**

<u>Factor</u>	<u>Number of Cases (%)</u>
Pregnancy Related	63 (35)
Additional Toxic Exposures	27 (15)
Infections	31 (17)
Surgery	11 (6)
Others	49 (27)
excessive dieting	
combat duty	
etc.	

Because the lungs are the site of beryllium toxicity, I thought it would be appropriate to review some of the changes in pulmonary function which occur during pregnancy. There is no difference in the respiratory frequency at rest or during exercise in pregnant and nonpregnant women. There are, however, significant increases in expiratory minute volume and tidal volume during pregnancy. At the present time it is not known if the increased toxicity of beryllium in pregnancy is a result of impairment of these pulmonary functions.

METALS AND FEMALE REPRODUCTION

I recently reviewed the literature on the effects of metals on female reproductive function in humans and experimental animals and was surprised to find a body of evidence which strongly suggests that many metals are indeed reproductive hazards (Mattison, 1983; Mattison et al., 1983). Reports from experimental studies in animals have allowed me to tentatively identify the site of toxicity along the reproductive tract (Table 5). Of the metals studied, cadmium, lead, and mercury are toxic at multiple sites along the reproductive tract of experimental animals. In addition, these metals are also toxic to human reproduction.

TABLE 5. EFFECTS OF METALS ON FEMALE REPRODUCTION^a

Site of Action	Metal									
	Lead	Cadmium	Lithium	Mercury	Chromium	Nickel	Selenium	Copper	Arsenic	Plutonium
<u>Experimental</u>										
<u>Animals:</u>										
Developing reproductive system	+	+	0	+	0	0	+	0	+	0
Puberty/sexual maturation	+	+	0	0	0	0	0	0	0	0
Mature reproductive system										
Hypothalamus-pituitary	+	+	0	+	0	+	0	+	0	0
Ovary	+	+	+	+	0	+	0	+	+	+
Uterus	+	+	0	+	0	+	+	+	0	0
Preimplantation events	+	+	0	+	0	+	0	+	0	0
Implantation	+	+	0	-	0	+	0	+	+	0
Resorption/embryonic death	+	+	+	+	+	+	+	+	+	0
<u>Human</u>										
<u>Epidemiology:</u>										
Fertility & spontaneous abortion	+	+	+	+	0	0	+	+	+	0

^a Taken from Mattison et al., 1983. + = Report of adverse effect; - = report of no effect; + = both positive and negative effects reported; 0 = no data.

Human studies are obviously less flexible - involving predominantly epidemiological observations with no clinical studies. However, when a metal has been studied and found toxic to reproduction in experimental animals it has also generally been observed to be toxic to reproduction in humans. With the exception of nickel, if a metal is toxic to two or more reproductive processes in experimental animals it is also suspected as a reproductive toxin in women.

LEAD

Inorganic lead was one of the earliest reproductive toxins identified in humans, and occupational exposure to inorganic lead also appears to impair fertility and increases the frequency of spontaneous abortion and stillbirth (Mattison, 1983; Mattison et al., 1983). Lead can also alter testicular and ovarian function. Men exposed to organic lead are more likely to have impaired libido and impotence compared to nonexposed men. In addition, occupational exposure to inorganic lead impairs spermatogenesis, producing clear dose-dependent impairment of sperm motility, density and morphology, with increasing levels of blood lead (Lancranjan et al., 1975; Levin, 1983).

ABNORMAL MENSES

The human ovary also appears to be sensitive to lead toxicity (Mattison, 1983; Mattison et al., 1983). About 25% of control women reported abnormal menses. This figure contrasts with the 45% of lead exposed women who report abnormal menses. Experimental animal studies suggest that although lead may act at more than one site along the hypothalamic-pituitary-ovarian axis, the major site of action is probably the hypothalamus or pituitary. Several investigators have demonstrated alterations in the secretion of FSH following lead treatment. Others have demonstrated alterations in ovarian and uterine function following lead treatment. All of these alterations in reproductive function seen in experimental animals are consistent with the observed reproductive abnormalities in humans.

DIBROMOCHLOROPROPANE

This small halogenated hydrocarbon, 1,2-dibromochloropropane, which is used as a soil fumigant by fruit growers, has been identified as a potent testicular toxin in humans and experimental animals (Pharoah et al., 1977). Interestingly, men appear to be much more sensitive to the testicular toxicity of dibromochloropropane than several strains of experimental animals.

DBCP EFFECTS ON EXPERIMENTAL ANIMALS

An early investigation by Torkelson demonstrated the dose-dependent decrease in testicular weight in rats, rabbits, and guinea pigs following treatment with dibromochloropropane (Torkelson et al., 1961). These and other similar experiments

were used to set occupational exposure levels in the United States. Unfortunately, the permissible exposure levels were set too high, and testicular toxicity has occurred following exposure to what was thought to be a safe level. Men exposed to dibromochloropropane for less than 3 months have normal sperm density in ejaculates. Those men exposed between 1 and 3 years have more than a 75% reduction in sperm count; those exposed for more than 3 years are essentially azospermic. Although many men exposed to dibromochloropropane for more than 3 years appear to be permanently sterile, many of those exposed for shorter periods, or to lower levels, appear able to resume spermatogenesis. Among farm workers exposed to dibromochloropropane in the fields, spermatogenesis remained suppressed for approximately 1 year following exposure, with recovery generally occurring between 18 and 21 months.

CHEMICALS IN BREAST MILK

Many women are not only working longer in pregnancy, but are also returning to the workplace shortly after the birth of their child. That suggests the need to consider the effect of workplace exposures which may be carried home to the infant by the mother. One potential source of exposure for an infant may be through breast milk. Table 6 summarizes the ratio of maternal

TABLE 6. RATIO OF CHEMICAL CONCENTRATION IN BREAST MILK (WHOLE BASIS) TO THAT IN MATERNAL BLOOD (M/P RATIO)

<u>Chemical</u>	<u>M/P Ratios</u>
Salicylate	0.35
Lithium	0.40
Caffeine	0.50
Theobromine	0.80
Theophylline	0.70
Phenobarbital	0.70
Methadone	0.80
Ethanol	0.8-1.0
Antipyrine	1.0
Mercury	
United States	0.90
Japan	0.10
Iran (organic)	0.03
Lead	<1
Tetrachlorethylene	~3
Polybrominated biphenyls (PBB)	~3
Polychlorinated biphenyls (PCB)	4-10
Dieldrin	~6
BHC	4-5
DDT residues	6-7

From Wolff, 1983.

milk concentration to maternal plasma concentration for a series of chemicals (Wolff, 1983). First note that all of the compounds studied were present in breast milk. Many of the chemicals listed have milk to plasma ratios less than 1. The halogenated organics, however, have ratios greater than 1. That is of some concern because many of these halogenated organics can accumulate in fat and remain in the body for many years, building up large loads in fat which are then passed via milk to the infant.

Calculations of the intake of several organohalides by infants based on typical levels in human breast milk and infant milk consumption suggests that the amount ingested exceeds the allowable daily intake level set by the Food and Drug Administration by 5 to 14 times (Wolff, 1983). Unfortunately, we do not know if this has an adverse effect on the infant.

COMPARISON WITH ANIMAL TESTING

In concluding this discussion of the adverse effects of workplace exposures on reproductive function, I want to stress the utility of animal models. We need to be able to predict reproductive hazards in order to prevent human reproductive disease. The existing data suggest that experimental animals are useful models for predicting potential reproductive hazards (Barlow and Sullivan, 1982). Benzene, toluene, and styrene all produce estrous cycle disturbances in experimental animals and menstrual disorders in women. Chlordecone, chloroprene, and dibromochloropropane all produce testicular toxicity in experimental animals and reproductive toxicity in men. Finally, arsenic, carbon monoxide, and polychlorinated biphenyls produce fetal toxicity or lethality in experimental animals, and are fetal toxins in humans. In spite of species differences in reproductive biology, the agreement between animals and humans suggests that data from experimental studies are clearly useful in identifying potential human reproductive toxins.

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ANIMAL MODELS FOR ASSESSING MALE REPRODUCTIVE TOXICITY¹

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Concern about the susceptibility of the male reproductive system to environmental agents is relatively recent. Much of this interest was generated by studies of workers exposed to dibromochloropropane (e.g., Whorton et al., 1979). In this instance reduced fertility, as a result of oligozoospermia, was present without any other clinical signs of toxicity. These findings suggested that for certain agents the reproductive system could be the first or most sensitive target organ. Besides the issue of fertility, there is the prospect of paternal exposure contributing to preimplantation or fetal loss, birth defects, childhood cancer, or neurobehavioral deficits.

The task of assessing reproductive risk in human populations is complicated by several factors. First, much of the information on sperm integrity (i.e., sperm count, viability, morphology) has been obtained from suspected infertile or subfertile men. In studies of presumably healthy, fertile men, limited data are available relating sperm integrity to sperm competence (i.e., fertilizing ability, successful pregnancies). If such information is lacking for fertile, normal populations, it follows that the impact of environmental agents on sperm integrity or competence is even more obscure.

Even assuming a defined relationship between sperm integrity and sperm competence, the study of environmentally-exposed human populations is fraught with problems. Gaining access to populations may be difficult. In addition, large numbers of individuals are required to detect exposure-related alterations in reproductive outcomes (e.g., malformations, early fetal loss). Additional difficulties relate to the individual's simultaneous exposure to multiple agents and uncertainty in defining actual exposure levels. Finally, the opportunity to conduct prospective, long-term studies relating exposure to semen status and concurrent reproductive activities (outcomes) is rarely afforded. Yet this is the ideal design in which to study these relationships.

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As a result of the restrictions encountered in the conduct of human reproductive studies, data from animal studies may have to be used to identify potential chemical hazards and to suggest exposure thresholds for adverse effects. Such data would ideally aid in the design of subsequent human studies as well as the delineation of mechanisms underlying the human response. However, most of the existing animal studies have evaluated only reproductive outcomes and not sperm integrity. Yet, it is the latter that provides endpoints most readily assessed in the human population.

Some of the parameters that can be evaluated in animal studies of the male reproductive system are listed in Table 1.

**TABLE 1. PARAMETERS THAT CAN BE EVALUATED
IN MALE REPRODUCTIVE STUDIES**

LIBIDO AND POTENCY

Endocrine primary lesions
CNS (non-endocrine) lesions

EVALUATIONS OF THE REPRODUCTIVE SYSTEM

Measures of sperm characteristics (concentration, viability, motility, morphometry, fertilization potential)
Biochemical markers (enzymes, proteins, unscheduled DNA synthesis)
Histopathologic assessment
Status of accessory organs and hormone analysis

FERTILITY AND FETAL OUTCOMES

Infertility
Pre-, postimplantation loss
Fetal viability and survival
Postnatal status
Survival
Growth
Functional deficits

Libido and potency have been traditionally evaluated in an indirect manner. Males and females are placed together overnight, and the presence or absence of a copulatory plug is determined the following morning. Occasionally, a vaginal wash is performed to establish the presence of sperm. The presence of a copulatory plug (or sperm in the vaginal wash) does not insure that the copulatory process is unaffected, but only that during the night the male was able to achieve at least one ejaculation. The sole means of evaluating the functional significance of CNS dysfunction on reproductive performance is to monitor the copulatory sequence.

Data from this laboratory on trichloroethylene (TCE) and carbon disulfide (CS₂) have shown that alterations in copulatory behaviors can be the earliest indicators of toxicity among the reproductive parameters examined. These effects are discussed later in this paper. Interestingly, both of these agents have been associated with disturbances in sexual dynamics in occupationally exposed workers.

The second category of Table 1 lists measures related to the structural and functional integrity of the reproductive organs themselves. The predominant measure employed has been histopathologic evaluation of the testes. This endpoint is important in providing insight into site(s) of insult. However, such techniques are not applicable to the study of human populations. Furthermore, the lack of testicular lesions does not preclude reproductive impairment. For example, secretions of the accessory glands nourish and maintain the spermatozoa. Thus, an impairment of the accessory organs could affect sperm integrity.

The number of studies in which sperm evaluations have been conducted is limited. Such evaluations are usually performed on sperm recovered from the cauda epididymis at time of sacrifice. Endpoints assessed include sperm count and morphology, and occasionally, sperm motility. The predominant sperm test in non-human mammals has been the mouse sperm morphology test used as an indicator of germ cell mutation. However, its utility when applied to environmental agents and/or tested in other species remains unconfirmed. The marked absence of evaluation of other parameters of spermatogenic dysfunction is best documented in a recent review by the EPA Gene-Tox Program (1983).

The final category of fertility and fetal outcome noted in Table 1 has been primarily evaluated using one of two strategies. Namely, the FDA - 3 tier protocol (Figure 1) or the multigeneration test protocol (Figure 2) expounded by EPA. Since the latter strategy entails simultaneous exposure of males and females, paternal versus maternal influences cannot be distinguished. An additional approach entails tests for germ cell mutation, exemplified in mammalian systems by the dominant lethal test. These tests are designed primarily to screen agents for their ability to cause embryonic death via germ cell mutation.

In summary, there are not any existing standardized testing strategies to evaluate the function of the male reproductive system, in terms of either fertility or fetal outcome, for agents whose primary mode of action is not germ cell mutation. Moreover, since most species produce a superabundance of sperm, fertility and fecundity are not particularly sensitive criteria for monitoring the reproductive process (Amann, 1982).

We have attempted to develop an animal model that will provide information on the overall function of the male reproductive system (Figure 3). As outlined earlier, data of this nature are not provided by other testing strategies.

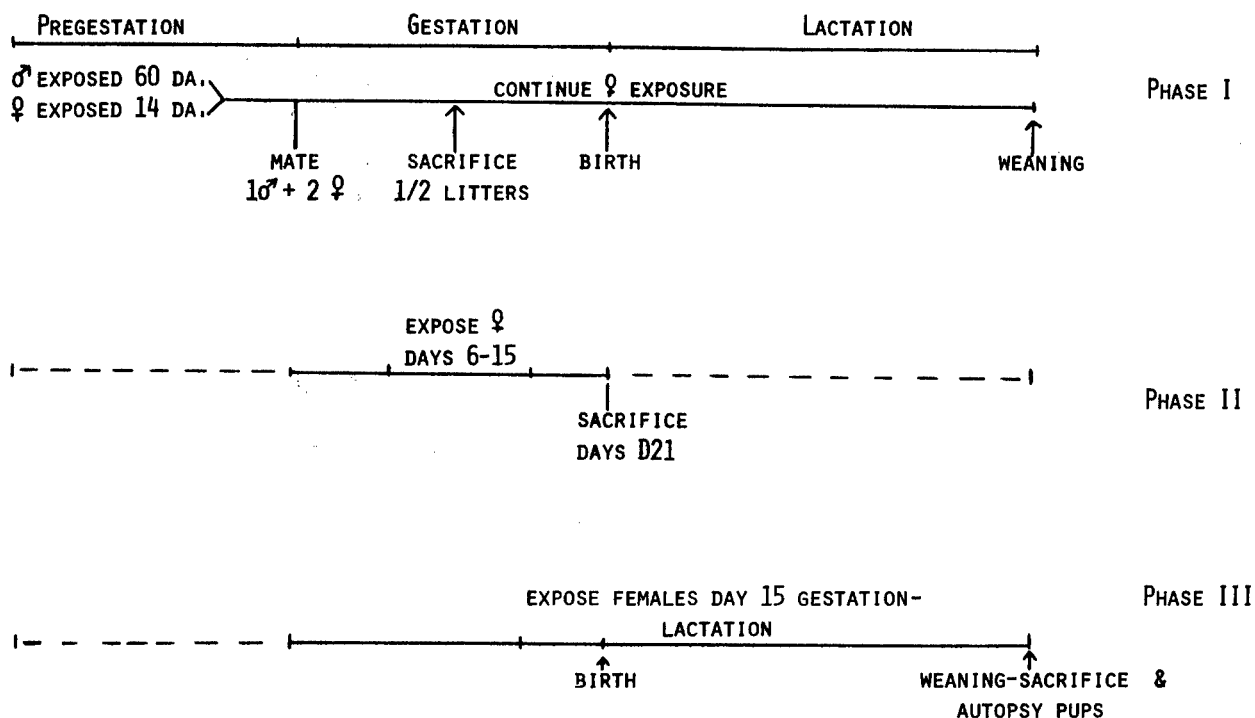


Figure 1. FDA 3-phase reproductive testing protocol.

In addition, we have emphasized endpoints which can be applied to studies of human reproductive function. The approach entails evaluating ejaculated semen samples recovered from the reproductive tract of a female rat at specified times post-copulation. This approach has a number of strengths:

- 1) The recovered ejaculate can be evaluated for semen parameters that may also be assessed in the human population.
- 2) For a given animal, a semen evaluation may be conducted prior to exposure. Repeated assessments may then occur during exposure as well as post-exposure (recovery). To this end, each animal may serve as its own control, enhancing the probability of detecting treatment-related alterations. This advantage can be best appreciated when one considers the inter-individual variability associated with semen evaluations. Moreover, a clearer picture of the degree of recovery for a given animal can be ascertained.

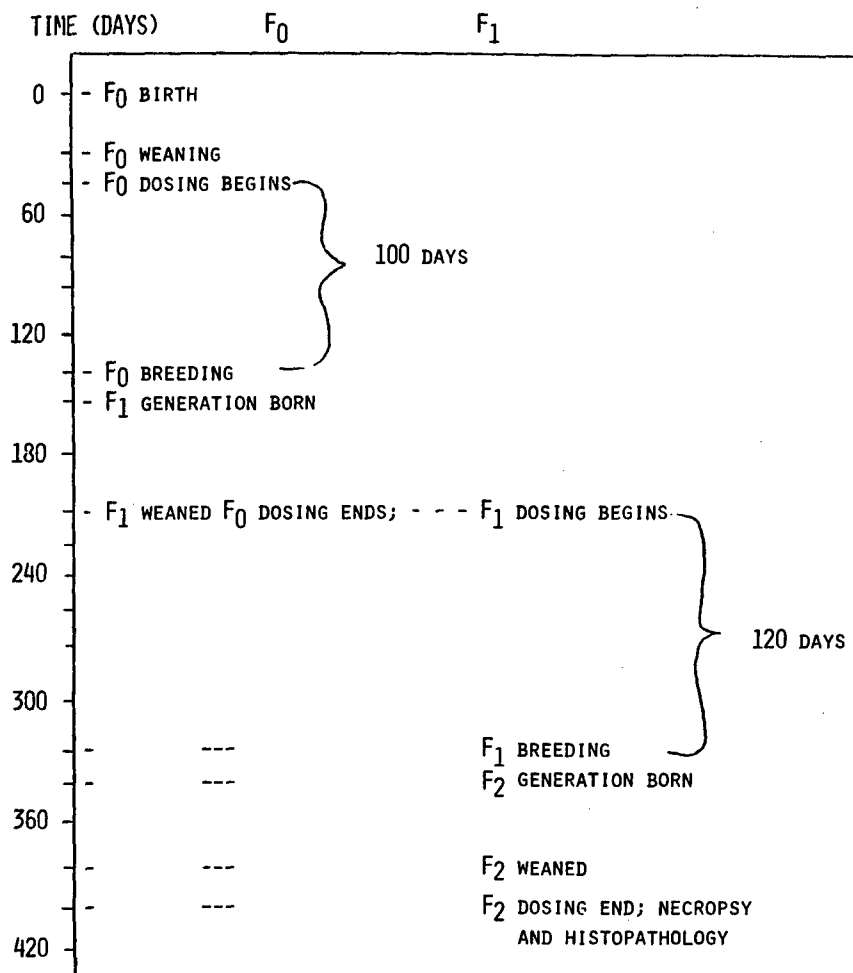


Figure 2. Multigeneration reproductive testing protocol.

- 3) Serial matings can be conducted concurrent with semen evaluations so that changes in the functional integrity of the sperm and reproductive competence can be correlated.
- 4) Traditional methods of semen recovery in rodents (e.g., electro-ejaculation) are stressful and may not be repeatedly applied. Our strategy of examining ejaculates recovered from the reproductive tract of a receptive female avoids this problem. The design also allows us to obtain data on copulatory behavior and sperm integrity while preserving the most natural environment in which the sperm may reside until evaluation.

This model shown in Figure 3 has been applied to the study of a number of compounds including TCE, 2,4,6-trichlorophenol, CS₂, and 2-ethoxyethanol (2-EE). We have also used this approach to monitor unscheduled DNA synthesis (UDS) in the ejaculate following exposure to methylmethane sulfonate (MMS). In the remainder of this paper we will describe some of the aspects of

the model and present normative and treatment-related data validating its utility. Data are also presented on our attempts to better quantitate certain semen parameters (e.g., motility) by using a computer-analyzed, videomicrographic scoring system. This system is also being used to evaluate human semen samples (Katz and Overstreet, 1981).

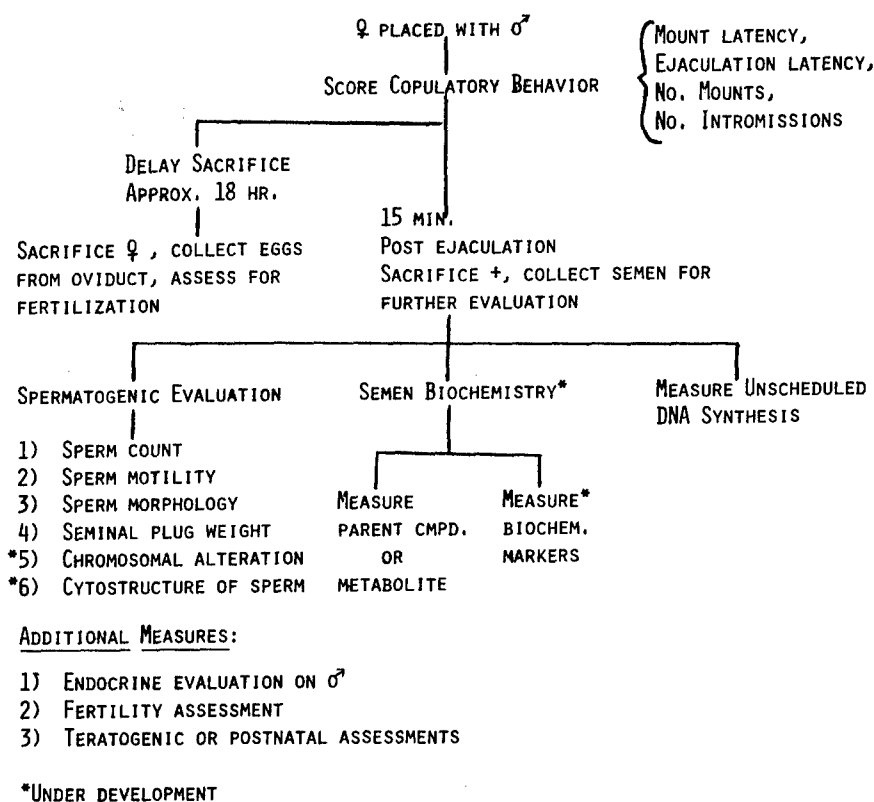
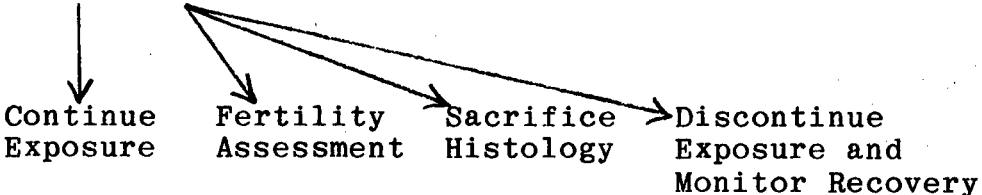


Figure 3. Protocol and parameters sampled in repeated measures model.

The timetable routinely employed in conjunction with this model is indicated in Table 2. Male and female rats (Long Evans hooded, Charles River) are introduced into the laboratory at 70 days of age. Females are then ovariectomized and allowed a one-to-two week recovery period. Receptivity is induced by an injection of estradiol 48 hrs prior to mating (0.1 mg/ml), followed by an injection of progesterone (0.1 mg/ml) 4 hours before mating. Initial investigations have found no differences in semen characteristics in samples recovered from intact, estrous females as contrasted to ovariectomized, hormonally-primed animals.

TABLE 2. TIMETABLE

AGE	
70 Days	o Introduced into laboratory Mated weekly with o to obtain mating proficiency
100 Days	Obtain baseline on copulatory behaviors and semen parameters
107 Days	Begin exposure
114 Days	Evaluate Wk. 1
135 Days	Evaluate Wk. 4
156 Days	Evaluate Wk. 7
177 Days	Evaluate Wk. 10
	

During the initial 30-day period, males are mated several times in order to gain proficiency in copulatory behavior. At 100 days of age, a baseline evaluation of sperm parameters and copulatory behaviors is conducted. Males with extremely low sperm counts (< 20 million/ml) or protracted ejaculation latencies (> 20 minutes) are eliminated from study. The remaining males are ranked on these two variables and assigned to control and treatment groups in such a manner as to produce comparable distributions across groups prior to initiating treatment.

The initial evaluation of semen and mating behavior is performed at 100 days of age, since at this time sperm production has reached its maximum adult level (Saksena et al., 1979). Studies that initiate exposure at 60-70 days of age risk introducing confounding factors attributable to the differing sensitivity of immature animals to insult. Insults incurred at this age may be quite different from those seen in the mature adult.

Because of the periodicity of the spermatogenic cycle, an acute (5 days) treatment regimen can be used to pinpoint effects on specific spermatogenic stages. Semen evaluations are conducted at one, four, seven, and ten weeks postexposure. Effects seen at specific time points would be indicative of damage to the spermatozoa, spermatid, spermatocyte, or spermatogonia stage, respectively. Subchronic exposures are usually for 70-80 days, corresponding to one full cycle of spermatogenesis in the rat. Several options may be pursued at the end of this period, includ-

ing sacrifice accompanied by histologic evaluation, traditional fertility testing, and/or monitoring of recovery. Males are mated weekly, even when semen evaluations are not conducted, in order to insure maintenance of a constant abstinence period.

As can be seen in Figure 3, the first component evaluated is copulatory behavior. The male is placed into a Plexiglas® observation chamber and given a 15 minute adaptation period. All observations are conducted under red light and during the dark phase of the light-dark cycle. The female is subsequently introduced and four behaviors scored: 1) mount latency (the interval between the introduction of the female and first mount), 2) the number of mounts, 3) the number of intromissions, and 4) ejaculation latency (the interval between the first mount and ejaculation). The occurrence of ejaculation in a rat is easily confirmed by presence of a seminal plug in the female tract at sacrifice. As noted earlier, two of the compounds we have studied to date, TCE and CS₂, alter copulatory behavior. A single TCE treatment (1000 mg/kg, p.o.) produces a protracted ejaculation latency which may be consistent with its purported narcotic properties. To this extent treatment with the narcotic antagonist, naltrexone (10 mg/kg, i.p.), reverses this behavior. Moreover, daily administration of TCE (1000 mg/kg, p.o.) produces comparable narcotic effects in the initial weeks of treatment. However, the effect on copulatory behavior is absent by five weeks of exposure. This phenomenon may be comparable to tolerance observed with continuous narcotic injections.

Daily CS₂ exposure (600 ppm, inhalation) produced an opposite effect, namely a significant decrease in ejaculation latencies seen by the fourth week of exposure (Zenick et al., 1984). These same males exhibited declines in ejaculated sperm counts in subsequent weeks. Interestingly, cauda epididymal sperm counts were not altered in these animals. It is possible that CS₂-induced alterations in copulatory behavior (e.g., premature ejaculation?) may have affected the number of sperm ejaculated (Chester and Zucker, 1970). Alternatively, CS₂ exposure may interfere with contractility of the vas deferens, reducing the number of sperm ejaculated. The important point is that conventional approaches which examine only cauda epididymal reserves would not have detected this effect.

Subsequent to copulation, the female remains undisturbed for 15 minutes and then is sacrificed (CO₂ asphyxiation). If intact estrous females are employed, sacrifice can be delayed for eighteen hours, and the eggs recovered from the ampulla and examined for signs of fertilization. To recover the semen sample, an abdominal incision is made, the reproductive tract exposed and the uterine contents withdrawn into a syringe (37°C). The tract is then excised, the seminal plug removed, rinsed, weighed, and the remainder of the seminal fluid flushed from the tract. Seminal plug weight is an easily obtained measure and may serve as a marker of hormone status since it is a product of androgen-dependent glands. An evaluation of Long Evans hooded,

male rats at approximately 120 days of age shows a mean seminal plug weight of 0.103 ± 0.021 gm (range 0.032 gm to 0.188 gm). As animals mature, seminal plug weights generally increase.

An aliquot of the syringe contents is diluted with culture medium (37°C) to provide a sample dilute enough to be scored for motility. The sample is then placed on a slide and several frames videotaped. The tape can then be played back for subsequent evaluation of motility.

A 15 ul aliquot of the diluted sample is smeared on two microscope slides, which are air-dried and stained with a combination of eosin Y, fast green, and naphthol yellow (Bryan, 1970). These slides are used for morphologic evaluations. In our experience, the LEH rat shows less than 3% abnormal sperm in both ejaculated and epididymal samples.

Total sperm number is obtained by rinsing all microscope slides, syringe contents, and tract washings with distilled water and diluting to a standard volume (50 ml). Aliquots of this dilution are loaded in both chambers of a Neubauer hemocytometer and two cell counts are performed.

The videotape is evaluated for percent motile sperm. In addition, fifty sperm in various segments of the tape are scored for swimming pattern and distance travelled (absolute distance and linear distance). The distance variables are measured using a digitizing cursor to track the sperm. These data are fed into a computer along with elapsed time to generate swimming speeds (microns/sec). A frequency distribution of swimming speeds and patterns is then generated for each male along with average swimming speed (data not shown). We feel that this approach maximizes the data generated from a semen sample. The distribution of each animal may then serve as a "sperm print" to contrast against the normative data base. Theoretically, specific exposures (xenobiotics, diseases, pathology, etc.) could be identified by such sperm profiles. However, this theory remains speculative until sufficient data are generated from future studies.

Data from a recent investigation of 2-ethoxyethanol (2-EE) can be used to illustrate the application of our model in evaluating various sperm parameters (Oudiz et al., 1984). In that study, a baseline evaluation was conducted; male rats then received either 0, 936, 1872, or 2808 mg/kg (p.o.) of 2-EE for five consecutive days. The males were mated weekly for the next 14 weeks. Semen evaluations were conducted on weeks 1, 4, 7, 10, and 14. The latter time point was included because males had begun to show recovery by the tenth week postexposure.

Data analyses indicated that 2-EE produced a rapid decline in sperm counts in the two highest groups, with most of the males becoming azoospermic by week 7. The males in the low dose group also exhibited a significant decrease in sperm counts at this week (Figure 4). Additionally, there was a significant increase

in abnormal sperm morphology at week 7 (degenerative sperm heads) and some depression in motility. Partial recovery was apparent in the semen analyses by week 14, as evidenced by an increase in sperm counts, and further supported by epididymal and testicular histologic assessments at week 16.

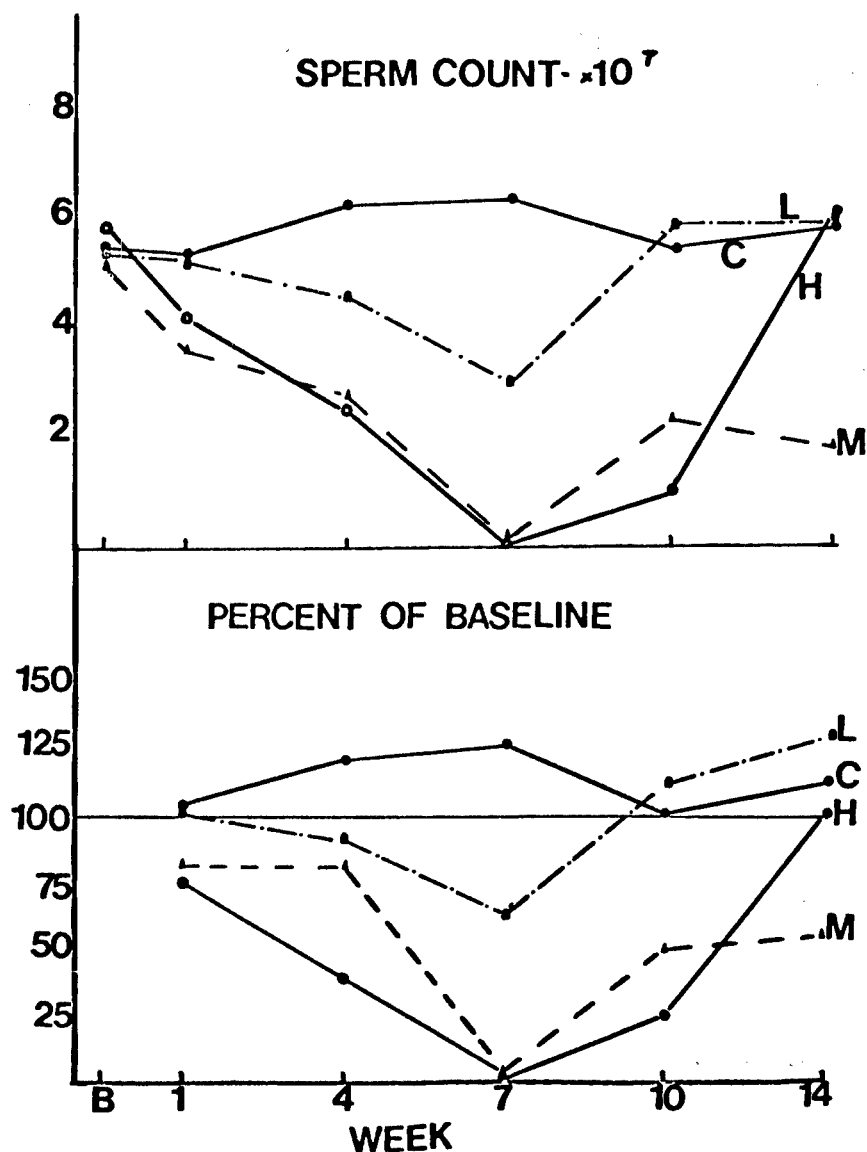


Figure 4. Changes in sperm count in male rats following a five-day exposure (p.o.) to 936 (L), 1972 (m) or 2808 (H) mg/kg of 2-ethoxyethanol.

Another example of the utility of this model is reflected in an investigation wherein unscheduled DNA synthesis was measured in ejaculates following treatment with methylmethane sulfonate. Rats received an intratesticular injection of ^3H -d-thymidine followed immediately by an injection of MMS (50 mg/kg, i.p.). Semen samples were then collected weekly for 10 weeks. The sperm heads were sheared from the tails by sonication; the heads were then separated by centrifugation. Radioactivity in the heads was

determined by liquid scintillation counting. In controls, radioactivity was only detected at 8-10 weeks postexposure, which corresponds to cells that would have been mitotically-dividing spermatogonia 8-10 weeks earlier (Figure 5).

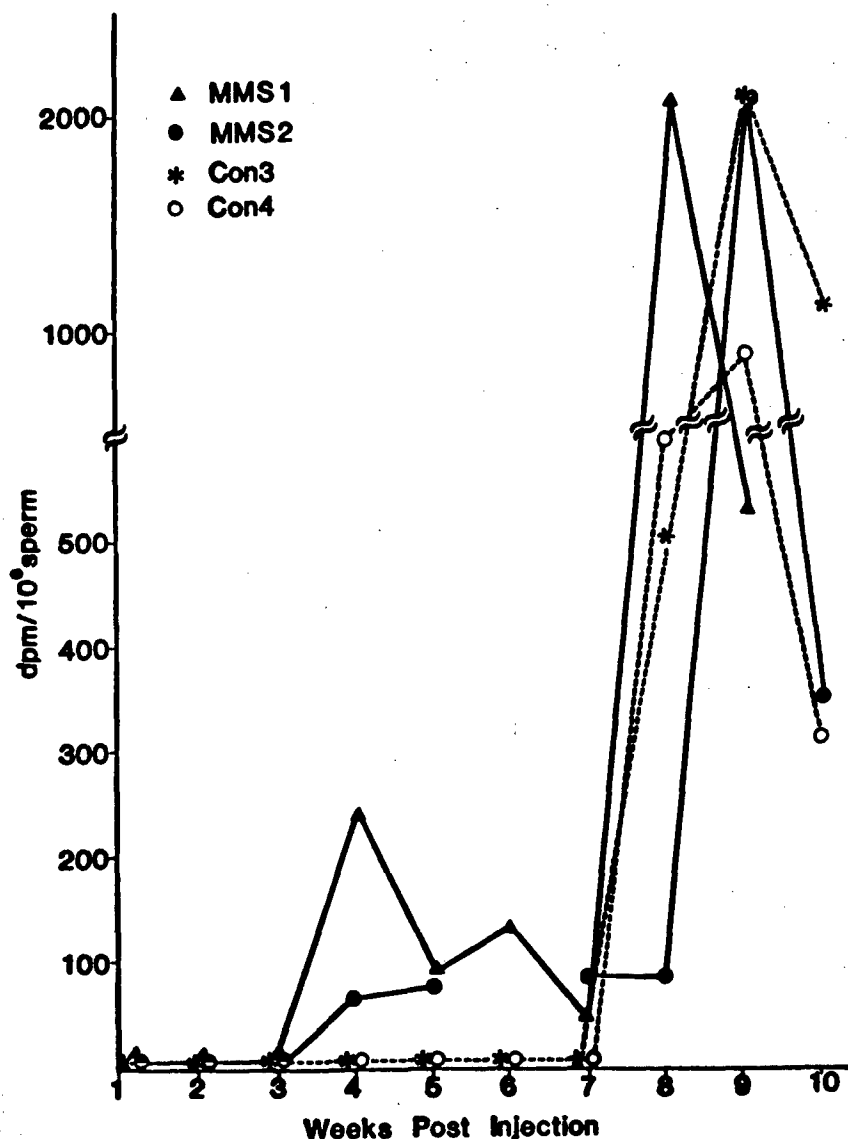


Figure 5. Levels of unscheduled DNA synthesis seen in ejaculated sperm following a single injection of methyl methane sulfonate (50 mg/kg, i.p.) Con = control animals.

MMS-treated animals also showed elevated levels of activity at these times. However, MMS also produced an increased incorporation of label between weeks 4-6 (early spermatid stage). This study serves to illustrate the point that the use of this model allows one to obtain corollary information on the germ cell without compromising the animal. The fact that UDS does not follow identical time courses across animals reinforces the utility of being able to follow an individual animal over time.

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HUMAN REPRODUCTIVE RISK ASSESSMENT FROM RESULTS OF ANIMAL STUDIES

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INTRODUCTION

The objective of this paper is to present a new approach to estimation of the hazard of toxic chemicals to the human male reproductive capacity, based on data that can be obtained from experiments on animals. Mechanisms by which an agent causes infertility will be considered and endpoints that can be quantitatively related to human infertility will be used. The approach is as follows. First, a dose-effect relationship for the agent and endpoint chosen in the experimental animal should be determined. Next, a corresponding dose-effect relationship on the same endpoint in man can be calculated using an "extrapolation factor," which relates that dose given to a test animal to the dose required to produce an equal effect in man. Finally, the alteration of the endpoint in man is used to calculate the increase in the incidence of infertility in the population.

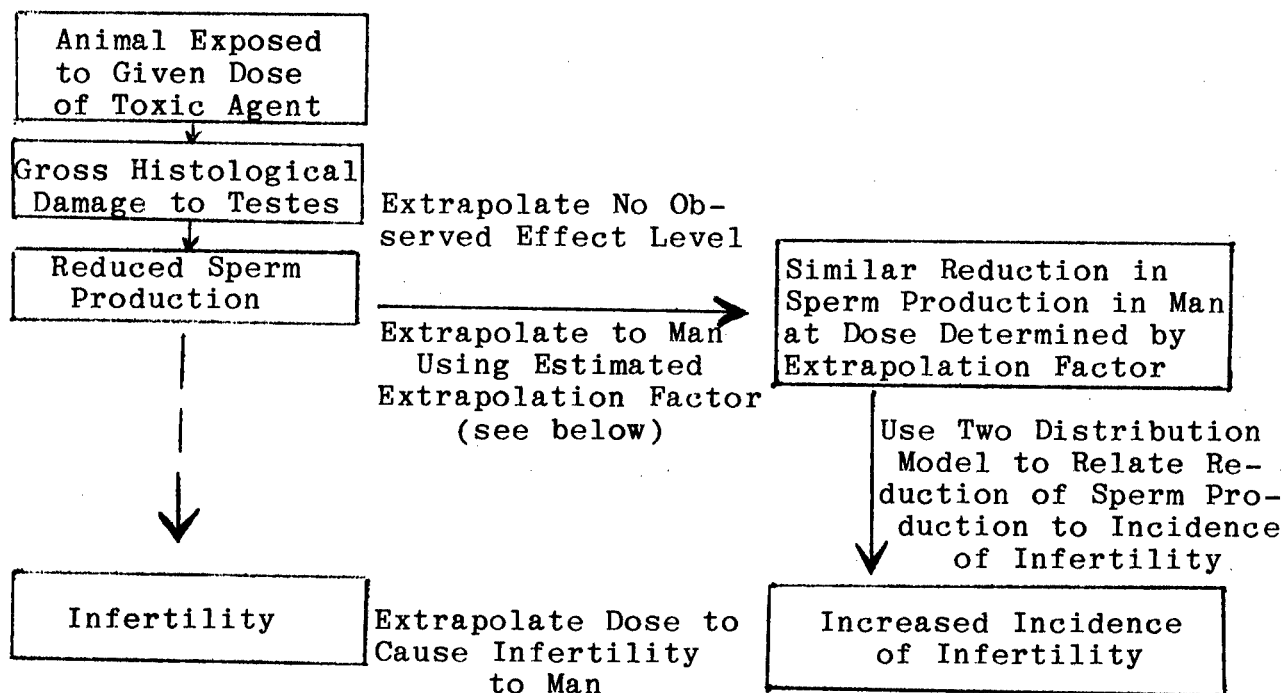
Although a variety of endpoints might eventually be used in this analysis, sperm production is the only one for which there are sufficient data for implementation of this method. The use of other endpoints such as gross testicular histology and fertility tests on experimental animals has been considered. I will show why sperm production is more desirable than these other endpoints for quantitative estimation of infertility in man based on data from experimental animals.

PROPOSED METHOD FOR EXTRAPOLATION OF ANIMAL DATA TO HUMAN MALE FERTILITY

The method suggested in this report for the extrapolation of the reproductive toxicity of an agent from experimental animals to man is outlined in Figure 1. In an experimental situation, animals are exposed to a toxic agent. Criteria for choosing a suitable measure for extrapolation are that the basic biology of the process in man and the test animals be similar and that there be a method for relating the alterations in that measured characteristic to infertility in man. Two conventionally used endpoints for assessing reproductive toxicity are histologic damage to the testis and infertility. (For reasons given in subsequent

sections, these endpoints have limitations for quantitative interspecies extrapolation of risk.) Sperm production shall be used as the endpoint here. Currently it is the only measure that can be quantitatively related to fertility in man. Furthermore, the sequence of spermatogenesis is quite similar in the experimental mammals employed and man, and hence sperm production fulfills the criterion of involving similar biological mechanisms in the different species. However, it must be pointed out that the method can only be applied in cases where the endpoint chosen is totally related to the infertility (i.e. the only significant cause of the infertility) in the animal exposed to that toxin.

A. MODEL FOR ESTIMATION OF HUMAN REPRODUCTIVE RISK
FROM ANIMAL DATA:



B. CALCULATION OF EXTRAPOLATION FACTOR:

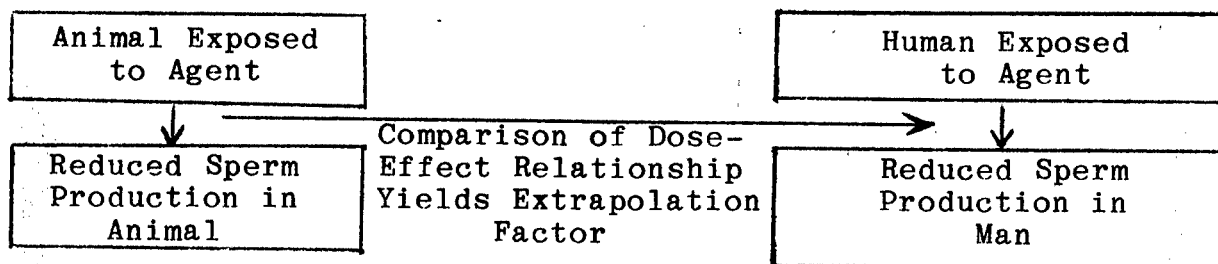


Figure 1. (A) Proposed method for extrapolation of data obtained on experimental animals to assess human male reproductive hazards is indicated by solid-line arrows. (B) Calculation of extrapolation factor based on data from related agents.

There are several methods of quantitating sperm production in experimental animals. Ejaculates can be obtained from some animals by either electroejaculation or by use of an artificial vagina, and sperm production computed from counts on ejaculates. A more accurate and simple procedure for determining sperm production is by quantitation of late spermatids in testicular homogenates (Amann, 1970; Mian et al., 1977). The dose-effect relationship for reduced sperm production in experimental animals should be obtained by this method.

Next, an "extrapolation factor" relating the dose required to reduce sperm production in an experimental animal and the dose to produce the same reduction in sperm production in man must be estimated. Interspecies differences might result in part from intrinsic differences in sensitivity between man and animals to the disruption of spermatogenesis. The remaining discrepancy would be a result of differences in transport, metabolism, and cellular response for a particular agent. A major problem has been to obtain comparable data in experimental animals and man on which to base extrapolation factors. Nevertheless, some data do exist and provide a basis for approximating extrapolation factors (Table 1).

Ionizing radiation is a model agent since variables such as transport and metabolism are eliminated. Results obtained with radiation show that the extrapolation factor can vary by as much as 12-fold depending on the time chosen for the comparison. At all time points chosen, man is more sensitive than the mouse, but the increase in sensitivity varies from 1.3- to 16-fold.

Sufficient data for calculation of extrapolation factors relating animal and human exposure to chemicals are available only for dibromochloropropane (DBCP) and cancer chemotherapeutic drugs. The extrapolation factor for DBCP is 20, not far from the range of values calculated for irradiation.

In extrapolation of the toxic effects of injected, implanted, or orally administered chemicals between species, choice must be made as to whether to use dosages by weight or by body surface area. Extrapolation had traditionally been done on a body weight basis. However, analysis of data obtained using animal lethality to anticancer drugs as an endpoint demonstrated that interspecies extrapolation of toxicity on a body surface area basis was superior (Freireich et al., 1966). Therefore, in Table 1, extrapolation factors for chemotherapeutic agents are given on this basis (mg/m^2). (For conversion to extrapolation on a weight basis, mg/kg , the factors would have to be multiplied by 11.) Man is somewhat more sensitive than mouse to chlorambucil (an alkylating agent) and MOPP (nitrogen mustard, vincristine, procarbazine, prednisone) combination chemotherapy. The extrapolation factors are within the range of those seen for radiation and DBCP. Adriamycin, on the other hand, demonstrates that man can be more resistant than an animal to the sterilizing effect of a chemical.

TABLE 1. COMPARATIVE SENSITIVITIES OF THE GERMINAL EPITHELIUM IN EXPERIMENTAL ANIMALS AND MAN TO TOXIC AGENTS

Agent (References)	Schedule and Route	Endpoint	Species And Dose Req.	Extra- polation Factor(a)
Ionizing Radia- tion (Clifton and Bremner, 1983; Rowley et al., 1974; Meisterich et al., 1978; Oakberg, 1959)	Single Dose	Reduction of type A sperma- togonia to 50% of control value at time of minimum	Human - 10 rad Mouse - 30 rad	3.1
		Reduction of sperm production to 50% of con- trol value at 1 year	Human - 50 rad Mouse - 800 rad	16
		Sperm production less than 50% of control value after maximum recovery	Human - 600 rad Mouse - 800 rad	1.3
Dibromochloropro- pane (Torkelson et al., 1961; Whorton et al., 1977)	Chronic inhalation (10-13 wks)	Significant testis weight loss and his- tological damage	Rat Rabbit Guinea Pig 5-12 ppm	
	Occup. Exp. including inhalation (8 years)	Reduction in sperm count to <1 million/ml during exposure	Human - 0.4 ppm	20
Adriamycin (Lu and Meistrich, 1979; daCunha et al., 1983a; Shamberger et al., 1981)	Single Injection	Reduction of SSI (b) to 10^{-3} permanent azoospermia(c)	Mouse - 50 mg/m ²	
	Monthly courses of chemotherapy (50 mg/m ² / course)	Azoospermia at 2 yrs. after end of therapy	Man - >500 mg/m ²	>0.1
Chlorambucil (Meistrich et al., 1982; Cheviakoff et al., 1973; Marina & Barcelo, 1979)	Single Injection	50% stem cell survival(d)	Mouse - 100 mg/m ²	
		Permanent azoospermia(e)	Mouse - ~2,200 mg/m ²	
	Daily oral admin. (2 - 10 mg/day)	Permanent azoospermia	Man - ~1,500 mg/m ²	1.5
MOPP Combination Chemotherapy (Meistrich et al., 1982; Sherins and deVita, 1973; deCunha et al., 1983b)	Single injection of single agents	50% stem cell survival (d)	Mouse - dose of drugs, equivalent to one course	
		Permanent azoospermia(e)	Mouse - 22 "courses"	
	10-day courses, repeated monthly	Permanent azoospermia(e)	Man - 4 courses	5.5

(a) Dose to produce effect in animal.
Dose for equivalent effect in man

(b) Stem Cell Survival Index (SSI) defined in Meistrich et al. (1978)

(c) A reduction of SSI to 10^{-3} corresponds to less than one surviving
stem cell per testis

(d) Measured by sperm head counts at 56 days after treatment (Lu et
al., 1980).

(e) A reduction of sperm production to 2×10^{-7} of control level
corresponds to an SSI of 10^{-3} (Meistrich, 1982) and hence per-
manent azoospermia. The dose to achieve this effect is calcu-
lated from the response to a lower dose assuming exponential
survival of stem cells.

The range of extrapolation factors for these agents, with the exception of Adriamycin, is between 1 and 20. When dealing with an unknown agent, an extrapolation factor would have to be chosen based on the greatest similarity to those in Table 1 with regard to mechanism of action and mode of exposure. In any case, use of an extrapolation factor of 20 should be a conservative choice.

The dose to man corresponding to a given reduction in sperm count can then be calculated using the appropriate extrapolation factor. If a sufficiently broad dose-effect relationship is available from an animal study, the whole response curve can be applied to man using the extrapolation factor; if not, it could be assumed that sperm production is an exponentially decreasing function of dose (Lu et al., 1980; Meistrich, 1982).

Next, a relationship between the reduction of sperm production and the increased incidence of infertility in a human population must be constructed. A method, which shall be referred to as the "two-distribution model," has been developed to provide such a relationship (Meistrich and Brown, 1983). The model assumes that sperm count is a good indicator of the fertility potential of a man. Distributions of sperm counts of men considered to be fertile and those in infertile marriages were compared, and the infertility risk for a man with a given sperm count was calculated from the ratio of these two distributions.

If exposure to a toxic agent reduces sperm count in all men by a factor "s", the increased incidence of infertility can be determined for exposed individuals as follows. The integral of the product of the sperm count distribution in exposed individuals and the infertility risk corresponding to a given sperm count is computed. These computations generate plots of the incidence of infertility versus "s" (Meistrich and Brown, 1983). The input distributions of sperm count reported by MacLeod and Gold (1951) and David et al. (1979) will be used with the understanding that in the latter case no semen analyses were performed on a fraction (assumed to be one-third) of the male partners of infertile couples because initial examination of the woman revealed a cause of infertility. The background incidence of infertility in the unexposed population will be assumed to be 15%. The increase in infertility in the exposed population is calculated under these conditions to be 4.2% for a two-fold decrease in sperm count. The increase in infertility is very nearly a linear function of the sperm count reduction factor for "s" less than 2. Thus, there is no threshold for increase in infertility as a function of reduction in sperm count in a population.

The assumptions and limitations of the two-distribution model have been presented (Meistrich and Brown, 1983). Although some of these assumptions require further testing, this model

will be used since it is the only one that can provide quantitative estimates of the increase in infertility in the human population.

EVALUATION OF GROSS TESTICULAR HISTOLOGY AS AN ENDPOINT

Gross histologic damage (or lack thereof) to the germinal epithelium of the testis is a commonly used endpoint in toxicologic investigations. However, it provides little quantitative information on which to base risk estimations.

One problem with this endpoint is that evaluation of the severity of the lesions is highly dependent on the quality of the preparations and the subjective judgement of the observer. If preparations are poor, damage must be more severe before it can be detected; since photomicrographs are not routinely shown, it is often impossible to determine the quality of the preparations. Furthermore, without quantitation, it is also difficult to judge the extent of the damage. Thus, a NOEL (no observed effect level) based on gross histologic analysis can be accepted only with considerable uncertainty as to the sensitivity of the assay.

Another problem is that there is no clear relationship between the level of qualitative histologic damage and infertility except in cases where the damage is severe. For example, if there were no late spermatids throughout the testis, the animal would be sterile, but when effects are less severe or extensive, no conclusion about fertility can be drawn.

Finally, gross histologic damage may be relatively insensitive for detecting toxic effects of chemicals. An example of this is provided by the previously published photomicrograph of a mouse testis 11 days after injection of 6 mg/kg of Adriamycin (Lu and Meistrich, 1979; Fig. 1a). This testis would be considered to be normal by gross histologic analysis; yet, cell identification and counts revealed a nearly complete absence of spermatogonia and an absence of pachytene spermatocytes in tubules at stages I through VI of the cycle of the seminiferous epithelium (Oakberg, 1956). These animals became sterile within a month after treatment and did not regain fertility until 102 days after treatment (Meistrich, 1982).

EVALUATION OF FERTILITY AS AN ENDPOINT

Fertility is another endpoint commonly reported in toxicologic studies. Although measurement of fertility is important, there are several reasons why fertility might not be an appropriate endpoint for extrapolation of reproductive risk from animals to man.

Firstly, the fertility of a male is a complex process and depends on numerous factors listed in Table 2. A toxic agent might exert its effect on any step in this process, and different steps may limit the fertility or be most sensitive to the toxin in different species.

TABLE 2. FACTORS INVOLVED IN MALE FERTILITY

<u>Factor</u>	<u>Subfactors</u>
Endocrine system (directly affects all sub-factors marked with * below)	Testosterone Luteinizing hormone Follicle stimulating hormone Gonadotropin releasing hormone
Sperm quantity	Proliferation of spermatogonial stem cells Differentiation of spermatogenic cells Function of Sertoli cells* Migration through epididymis and vas deferens*
Sperm quality	Genetic integrity Maturation in epididymis* Motility Ability to penetrate cervical mucus and egg vestments Ability to fertilize egg
Secretions of accessory sex organs	Seminal fluid* Coagulating substance* Prostatic secretion*
Sperm delivery	Ejaculation* Libido* Behavioral factors*

Secondly, even if the same target is affected by a toxin in two species (human and the test animal) resulting in the same degree of damage to that target in both species, it cannot be assumed that fertility would be similarly affected in both species. This is because the measurement of fertility is usually an insensitive endpoint for detection of agents that adversely affect spermatogenesis in experimental animals since most of these animals produce spermatozoa in vast excess of the number required for fertility. For example, a rabbit produces 2×10^8 sperm per ejaculate (Amann, 1981); yet, 2×10^6 are sufficient to achieve reliable fertilization by artificial insemination (Galbraith et al., 1983). The rabbit produces more motile sperm per ejaculate than man, a much larger species, and hence human fertility might be more drastically affected by a reduction in sperm counts.

Thirdly, depending on the particular endpoint being considered, the sensitivity of a fertility assay can vary widely. For example, the ratio of mated to pregnant females would be relatively insensitive while the ratio of unfertilized eggs to 2- to 8-cell embryos (obtained by flushing the oviducts) would be more sensitive. Evaluation of the sensitivity of the endpoint for fertility determination is usually not considered; this makes interpretation of a NOEL difficult.

Measurement of fertility is still very important in studies of reproductive toxins. First, as indicated in Table 2, there are many targets for reproductive toxicity; only assessment of fertility can screen for effects on all of these targets. Second, if both testicular effects and infertility are observed, it is important to know to what extent the infertility is related to the observed testicular damage. If the infertility is totally related to the testicular damage, then there should be no other damage produced, beyond that observed in the testis, that contributes significantly to the infertility. If they are unrelated, then the level of testicular damage is not sufficient to have any significant effect on fertility and the observed infertility is due to toxic effects on other targets. A partial relationship implies that the reduced fertility is partly a result of testicular damage, but that other factors also contribute to the infertility. Which of these three options applies has great significance for risk estimation. If the two endpoints are totally related, then the estimation of infertility risks in man could be based on extrapolation of reduced sperm production observed in experimental animals, as proposed here. If infertility in the test animals were only partly due to damage to the germinal epithelium, then man would be at greater risk than that calculated simply on the basis of reduced sperm production. If testicular toxicity in the experimental animal were unrelated to the infertility, extrapolation of reduced sperm production would most likely lead to a gross underestimation of the potential toxicity of the agent to reproduction in man. Thus, it is important to ascertain, through basic studies on experimental animal systems, the degree of interrelationship between the observed endpoint and infertility.

EXAMPLE OF APPLICATION OF THE PROPOSED METHOD

Unfortunately, most published toxicologic studies have not attempted to obtain data in the form that could be directly used in the analysis proposed here. An example of a potentially useful study is one of glycol ethers (Miller et al., 1983; Rao et al., 1983) in which rats were exposed to ethylene glycol monomethyl ether (EGME) and, at the end of the exposure period, testis weight, gross testicular histology, and fertility were measured, but sperm counts were not. Nevertheless, these data shall be used here to obtain a rough estimate of sperm production in order to obtain a measure that can be extrapolated to man. After exposure to 300 ppm, the testis weight was reduced to 41% of control, most tubules contained only Sertoli cells and few

spermatogonia, and very few tubules contained germ cells of all stages. At a lower dose (100 ppm) there was no significant loss of testis weight but the standard errors were such that, at the 95% confidence limits, a weight loss as high as 32% might not have been detected. After exposure to 300 ppm all animals were sterile; fertility was unaffected by 100 ppm.

Based on the marked effects on the testis at 300 ppm and the error limits in testis weight measurements at 100 ppm, I estimate the reduction of sperm production at 100 ppm to be 80% of control values. Of course, if sperm production itself had been measured it would be possible to obtain the desired value directly. Since the dose at which testicular damage is detectable corresponds to that at which sterility occurs, I shall assume that the infertility is totally related to the testicular damage and the reduced sperm production.

Next, an acceptable level of increased infertility in the exposed population, based on the benefits from the procedure that results in exposure, should be selected. An increase of 1% will be chosen arbitrarily in the example. The two-distribution model predicts that a 1.24-fold decrease in sperm counts would correspond to a 1% increase in infertility in the population. Since a 1.24-fold decrease represents 80% of control levels, 100 ppm can be used without further calculation as the dose to the rat to reduce sperm production to a level that would, in man, result in a 1% increase in infertility.

The extrapolation factor for comparing the dose in rat to that in man shall be chosen as 20, based on results obtained for DBCP. This choice was made since both agents were administered by inhalation and 20 was the highest extrapolation factor observed for any agent. Thus, 5 ppm is the estimate of the exposure level to EGME that would increase the incidence of infertility in the human population by 1%. In contrast, the traditional approach would have been to take 100 ppm as the NOEL in the rat and to choose a safety factor to estimate a dose at which there would be no observable effect on man. The shortcomings of this approach are as follows. As discussed above, the NOEL is very dependent on the sensitivity of the assay. Furthermore, the choice of the safety factor is somewhat arbitrary. Recently, a safety factor of 100 below the NOEL obtained when very sensitive assays were employed was suggested (Galbraith et al, 1983). This choice was based on the general observation that the human testis functions less efficiently than that of experimental animals, but not on any specific, quantitative data.

CONCLUSIONS

A new approach for human reproductive risk assessment based on results of animal studies has been presented. This assessment is more quantitative than previous methods. Although the quantitative approach contains some assumptions and estimations, these are clearly defined and can be further examined and tested. For

example, the calculated extrapolation factors are preliminary estimates. It is hoped that this analysis will serve to focus and stimulate the collection of more data on human exposure and direct researchers using experimental animals towards designing the appropriate experiments for comparison with man. Nevertheless, providing the uncertainties involved in the assumptions and estimations are relatively small, the approach presented here may be ready for immediate application to assessment of human male reproductive risk from certain types of toxic agents.

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METHODS FOR SURVEILLANCE OF REPRODUCTIVE HAZARDS TO HUMANS

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INTRODUCTION

There is a growing awareness that, in some situations, environmental hazards to male reproduction must be studied directly in exposed men as well as in animal models. The tests which are available for human reproductive surveillance are those which have been applied clinically in assessing male infertility (Overstreet, 1984a, b). There has been significant improvement of these clinical tests during the past ten years and several new tests have been recently developed. The application of this new technology for risk surveillance of reproductive hazards offers the possibility of greater accuracy in detecting early signs of human male reproductive toxicity. However, effective use of these tests for environmental assessment will also require an appreciation of their biological basis and clinical limitations.

SEMEN ANALYSES

Most tests of male reproductive function involve some type of semen analysis and these tests will be the principal topics for discussion in this paper. In the standard clinical evaluation of semen quality, the semen volume is measured, the concentration of spermatozoa in the semen is determined, and the motility and morphology of the sperm cells are assessed (Eliasson, 1975). A great deal of information on male reproductive function is contained in this standard semen evaluation.

Sperm Concentration. The most reliable clinical parameter of the semen quality has been the concentration of spermatozoa in the semen or the "sperm count". This parameter is usually expressed in terms of sperm concentration per ml of semen. The product of the sperm concentration and semen volume is the total number of sperm in the ejaculate. This number provides a better indication of sperm production by the testes than does the sperm count alone. However, clinical assessments of semen quality traditionally emphasize the sperm concentration (Eliasson, 1975), and alterations in semen volume are considered significant only when the volume is very low (less than 1.5 ml) or very high (greater

than 5 ml). In the past, clinicians have relied primarily on sperm counts because this parameter alone can be measured objectively (either hemocytometers or automatic counting devices can be used). Nevertheless, the "normal range" for the sperm count, usually considered to be 20 - 250 x 10⁶ per ml, is very broad and as many as 80% of infertility patients may meet the minimum standards for sperm concentration (MacLeod and Wang, 1979).

Sperm Motility. The necessity for taking account of other semen parameters in the clinical assessment of the male has long been recognized. The recent development of techniques for objective assessment of sperm motility has increased our confidence in the validity of these measurements and has allowed more effective use of this information in clinical assessments. Traditional methods for measuring sperm motility involve subjective assessment of the number of motile sperm (percent motility) and of the quality of sperm movement (a numerical rating of 0 to 4+) (Eliasson, 1975). More recently, objective methods for sperm motility assessment have been introduced for determining the percentage of motile sperm in the semen and their mean swimming speeds. The method currently in use in our laboratory involves video recording of sperm movement in the semen sample (Katz and Overstreet, 1981). The videotape is subsequently replayed and analyzed for sperm movement characteristics. The tape also serves as a permanent record of the semen quality for subsequent review and/or additional analysis, as needed. Sperm swimming speeds are measured from the video screen utilizing a transparent overlay with concentric rings which have been calibrated using a micrometer. The transparency is centered on the spermatozoon being assessed, the tape is advanced for a standard interval of time, and a swimming speed is calculated by reference to the ring on the overlay which the sperm has reached (Katz and Overstreet, 1981). We have used this technique routinely in our clinical laboratory for evaluation of subfertile semen.

Sperm Morphology. Changes in human sperm morphology are associated with many abnormalities of reproductive function in men (Eliasson, 1975). It has also been recognized for many years that alterations in sperm shape can reflect environmental stress to the human testes (MacLeod, 1974). However, the usefulness of this potentially valuable parameter has been limited by the absence of a standard classification system for human sperm morphology and by the imprecise, subjective nature of the morphology assessment process (Freund, 1966). We have also found videomicrography to be useful in assessing human sperm morphology. The spermatozoa are assessed from stained seminal smears. The images of the sperm cells are transmitted to a video screen which the laboratory technician views in making the morphology assessment (Overstreet et al., 1981). It is not necessary to make a video-recording unless an additional record is desired. A transparent overlay is also used for morphology assessment. The overlay is

calibrated with the metric standards for normal sperm dimensions and allows the classification of sperm head shape on the basis of objective metric standards rather than subjective impression (Katz et al., 1982).

THE APPLICATION OF OBJECTIVE METHODS FOR SEMEN ANALYSIS

The application of these methods for semen analysis is well illustrated in a pilot study we have recently completed on the effects of cigarette smoking on human semen quality (unpublished observations). Patients referred to our clinic for semen evaluation were interviewed to obtain medical and social histories of the husband and wife. The semen quality of 286 cigarette smokers was then compared with 284 nonsmokers. When the semen quality in the two groups was initially compared by analysis of variance, no significant differences were detected in any semen parameter. However, when all cases with known male or female reproductive pathology were excluded, a significant difference in sperm concentration was detected in the two groups (mean sperm concentration 102×10^6 per ml for nonsmokers [N=62] vs 68×10^6 per ml for smokers [N=73], $p < 0.05$). Regression correlation analysis with this selected group of smokers revealed a negative correlation of sperm concentration with pack years ($p < 0.05$), and percent normal sperm ($p < 0.01$). A positive correlation with pack years and percent tapering sperm (long considered an indication of "stress" to the testes; MacLeod, 1964) was also detected ($p < 0.01$). When the age of the patient was factored out by application of partial correlation analysis, the significance levels were unchanged. No effects on sperm motility were detected in this group of patients using the time exposure photomicrography method (Overstreet et al., 1979). An effect of tobacco on male reproductive function has been suspected for many years (Amelar et al., 1980). However, these data are the most clear in demonstrating such an effect. This can probably be attributed to the greater precision of the assay methods as well as more careful selection of the study population.

INTERPRETATION OF THE SEMEN ANALYSIS

Even our most sophisticated, objective, quantitative methodology can only provide a general assessment of whether the semen quality is "normal" and some idea of the degree of semen abnormality. Our current clinical assessments of what constitutes "normal" or "fertile" semen quality derive primarily from the classical studies of John MacLeod (MacLeod, 1971). MacLeod studied a large group of men whose wives were pregnant at the time of the semen evaluation, and compared their semen quality with that of a group referred for diagnostic semen evaluation because of an infertile marriage. On the basis of statistical distinctions between these groups, MacLeod set "normal standards" for semen quality which remain the most widely accepted criteria for the contemporary semen evaluation.

It must be appreciated that these values are not strictly analogous to the normal range of biological values which are employed for interpretation of many other clinical laboratory tests. Recently, there has been controversy concerning the value for the lower limit of normal sperm concentration in human semen. Studies of semen from previously fertile men seeking vasectomy have revealed many men with sperm concentrations lower than MacLeod's normal range (Nelson and Bunge, 1974; Zuckerman et al., 1977). Subsequent information on the seminal sperm concentrations recorded in MacLeod's laboratory during several decades (MacLeod and Wang, 1979) has diminished the possibility of a decline in semen quality in American men as others have speculated (Nelson and Bunge, 1974). Nevertheless, these discrepancies are almost certainly real and they emphasize the limitations of our current standards.

A cursory examination of the groups of men in these various studies reveals significant confounding factors which are seldom taken into account. In general, MacLeod's fertile group of men were of recent proven fertility, but they were also relatively young, primarily of Italian descent and an urban population of lower socio-economic status. It could be speculated that the pre-vasectomy populations studied may not have been comparable in terms of age, ethnic background, socio-economic status, occupation, sexual activity as well as proximity of proven fertility. The infertile groups studied by MacLeod and others (e.g., Zuckerman et al., 1977) were largely unselected patients referred for semen evaluation because of infertile marriages. Undoubtedly, many of these infertility cases had contributing female factors and many of the men were probably fertile. Such studies rarely attempted a followup of the patients to determine their ultimate fertility or the results of subsequent diagnostic tests on the male or female.

Similar large-scale comparative studies of fertile and infertile men have seldom been reported for the parameters of sperm motility and morphology. It seems likely that the same types of confounding factors would interplay in such studies and would probably be magnified by the subjectivity of the motility and morphology assessment as they are routinely performed. A confident prediction of male fertility potential on the basis of semen parameters alone will not be possible until objective methods for assessment are available and until we develop a better understanding of the "normal" semen quality and its variation with factors such as age, health, social status, occupation, etc.

Interpretation of the semen parameters in field studies of reproductive toxicity is also complicated by the fact that the "normal" values for these tests have been determined by comparison of groups of known fertile men with groups of infertility patients. Virtually all of the clinical experience with these tests has been with clinically infertile couples (i.e., trying to conceive without success for at least one year). There are no data available with which to calculate a true normal range for

any of the laboratory tests of human semen. This means that studies of reproductive toxicity must be carried out by comparison with carefully matched, non-exposed control subjects. Only in cases of very severe semen abnormality can any prediction of fertility potential be given for a specific individual in the absence of clinical infertility.

NEW TESTS FOR HUMAN MALE FERTILITY ASSESSMENT

Morphometric Assessments of Human Spermatozoa. Successful application of clinical methods for semen evaluation in human field studies will require an understanding of the potential artifacts which may confound the data being collected. Virtually all of the semen parameters are subject to such artifacts. Semen volume and sperm concentration may be altered (increased or decreased) by the interval of sexual abstinence as well as the process of semen collection. Sperm motility is relatively fragile and may be adversely affected during aging of the semen after collection and by variations in temperature during transportation or processing of the specimen. Sperm morphology is unaffected by these variables, and in field studies measurements of this parameter can be interpreted with the least concern for artifact. The previously described methods for assessment of sperm morphology by metric standards have substantially increased our confidence in this laboratory test. However, the type classification system (e.g., oval, large, small, tapering, amorphous, etc.) is cumbersome even when based on metric standards. Furthermore, there is no sound biological or clinical basis for the "normal values" of sperm dimensions which have been proposed for human spermatozoa (Katz et al., 1984).

An alternative approach to human sperm morphology assessment involves determination of a morphometric parameter for the semen specimen. Such a parameter is calculated directly from measurements of sperm dimensions. This approach is practical only with computer assistance. In our initial studies using this approach we have digitized the contours of spermatozoa from their images on the video screen by manual tracing with an electromagnetic digitizer (Katz et al., 1984). The digitizer is integrated to a microcomputer and coordinates of the sperm contour are directly processed for calculation of head length, width, circumference, area, etc. Our current approach is based on the observation that spermatozoa in fertile semen are fairly uniform in size and shape, whereas the semen of infertile men displays a greater diversity of sperm morphology (MacLeod, 1964). It is our hypothesis that the magnitude of this diversity can be descriptive of the degree of abnormality in semen quality. In a recent clinical study, we found that the number obtained after dividing the sperm head length by its width (aspect ratio) was the morphometric parameter that differed most between a group of 30 fertile men and a group of 30 infertile men. Moreover, it was the within specimen variability of this parameter (standard deviation) rather than the central tendency (mean value) which maximized the difference between the groups (Katz et al., 1984).

Tests of Sperm-Cervical Mucus Interaction. These tests have proven quite useful clinically since they measure some important functions of the sperm cell, including sperm motility and surface properties, which are related to sperm migration through the female reproductive tract (Overstreet and Katz, 1981). These tests also differ from those of the simple semen evaluation, since they assess the interaction of male and female components. In general, the results of these assays reflect the success of sperm penetration through the mucus, e.g., the time required for sperm passage through the mucus and/or the number of sperm cells which succeed in entering the mucus (Overstreet, 1984). The practicality of applying such tests for assessing toxicity to spermatozoa is currently limited since human cervical mucus is only available at specialized clinical centers. It is possible that preparations of bovine cervical mucus, which are commercially available, may be useful in surveillance of reproductive hazards to male fertility. However, the clinical experience with these tests (Alexander, 1981; Borghi and Asch, 1983) is currently insufficient to assess their diagnostic efficacy.

Tests of Sperm-Oocyte Interaction. Recently, there has been considerable progress in the application of in vitro fertilization for treatment of human infertility. However, the fertilization of human oocytes for diagnostic purposes is not ethically acceptable, and human oocytes are seldom available for laboratory study. The discovery that the zona pellucida is the primary block to interspecies fertilization in the golden hamster led to the use of the zona-free hamster oocyte as a test material for assessing functions of the sperm cell involved in fertilization (Yanagimachi et al., 1976). There have been a number of clinical reports which indicate a close association between human male fertility and the success of fusion with zona-free hamster eggs in vitro (e.g., Rogers et al., 1979; Karp et al., 1981). A failure of sperm fusion in the assay may detect deficiencies in the sperm cell which would not be apparent from other tests of male fertility (Overstreet, 1983). However, not all sperm functions are recognized by the test and a positive result therefore has limited clinical value (Gould et al., 1983). An additional impediment to application of the test in surveillance of reproductive toxicity has been its limited availability in specialized clinical laboratories. However, the recent development of methods for cold storage of semen for up to 48 hours (Bolanos et al., 1983) will permit shipment of specimens from distant field locations to a base laboratory for testing.

ASSESSMENT OF EARLY PREGNANCY LOSS

It is possible that some types of reproductive toxicity to the male may be expressed as post-fertilization reproductive failure, i.e., early spontaneous abortion in the female. The occurrence of early pregnancy loss ("occult pregnancy") may not

be apparent either to the affected woman or to her physician. Our ability to monitor for this type of reproductive hazard is dependent on the application of tests for detecting very early pregnancy.

Most clinical studies of early pregnancy have assessed the concentration of human chorionic gonadotropin (hCG) in blood or urine on the days preceding menses. In prospective studies of normal conception cycles, the rising titer of this hormone is apparent several days before the expected menses (e.g., Edmonds et al., 1982). However, the interpretation of the comparable data from abnormal pregnancies is fraught with difficulty. It is likely that the failing embryo will produce subnormal levels of the hormone and/or the time of appearance of the hormone may differ from the normal pregnancy (Edmonds et al., 1982). These considerations lead to uncertainty in the interpretation of endocrine profiles for cycles with suspected early abortion.

One recent study of normal women reported a spontaneous abortion rate of 62%; 92% of these abortions were detected solely by endocrine criteria (Edmonds et al., 1982). Studies in our laboratory have not supported this view (Hanson et al., 1984). In our prospective study of daily morning urine samples from 197 women in 203 menstrual cycles, 14.6% of 48 pregnancies terminated in clinically apparent spontaneous abortion. This result agrees well with the generally quoted rate of spontaneous abortion in 15% of recognized pregnancies (Roth, 1963). Urine from 100 apparent nonconception cycles was analyzed for the β subunit of hCG and in 14 of these cycles there were apparent elevations of hCG near the time of menses. However, when we looked for a biological response to hCG as indicated by an increase in steroid production by the ovary (Lasley et al., 1983), only three of the fourteen suspect cycles showed evidence of such a response (Hanson et al., 1984). Our interpretation, if correct, would suggest that the incidence of peri-implantation spontaneous abortion may be less than 5% in normal women.

In conclusion, the methodology for monitoring early pregnancy in women has currently reached an advanced state of development and has been applied in several clinical studies. There is currently some controversy regarding the interpretation of data obtained in these studies, but it seems likely that similar tests will be available for reproductive risk assessment in the near future.

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OPEN FORUM III

Dr. Tanaka (NIOSH): In our work we sometimes conduct semen sampling in industry wide studies for health hazard evaluations. My question is to Dr. Overstreet. You mentioned that we should avoid extremes of temperature. What's the upper limit and also lower limit allowed and what is the effect of freezing and thawing the semen sample?

Dr. Overstreet (University of California, Davis): There have been studies done on the effects of temperature on sperm motility and we are specifically talking about sperm motility as a sensitive parameter to temperature. I think to give absolute values would not be very meaningful because we have seen that there is a great deal of individual variation. It may well be that some toxic substances may increase the sensitivity of the sperm cell to a cold shock or heat shock. Ideally, one wants to maintain the semen sample at or close to body temperature during the time it is transported. The ideal way to achieve that is to have the individuals being assessed collect the semen sample at the laboratory. I realize that there are great logistic problems in setting up such a study although I think that there now is some operational research beginning on the feasibility of having a mobile laboratory which would visit the worksite at which individuals could collect the semen samples. If this is not possible, there are some alternative methods. One is to provide the subjects with an insulated container in which the subject would place the semen sample and then transport it to the laboratory themselves. In climates in which temperature is not terribly extreme, the best method probably is to ask the subject to carry the container in an inside shirt pocket under their outer garments. This will usually maintain appropriate temperature for the semen sample. I think that one of the things we need to do is to work out methodology for assessing the level of artifact in any samples that we are assessing in the field. We are interested in doing this and it may be that by looking at the relationship of the semen parameters, that is the relationship of the sperm motility to the sperm concentration and morphology in normal populations, we will be able to gain some idea of whether our data on sperm motility is indeed reliable or whether there is an artifact. I think that would be very helpful in assessing the data. This is the great limitation in using sperm motility assessments in field studies. As to the effect of freezing the sperm cells, that can be done for transportation of semen samples for making sperm counts but not for motility studies. It should not be done prior to making preparations for sperm morphology assessments. These can be made in the field with fresh semen samples. An alternative to freezing the sample in terms of returning it for sperm count is to dilute the sample in a fixative which can also be done easily in the field. That is, bringing small vials with pre-measured amounts of a normal saline mixture, diluting with a fixed amount of semen and then making

Dr. Meistrich (M.D. Anderson Hospital and Tumor Institute): Yes, that certainly is a good point. One example that we presented was adriomycin. We have no explanation for why the mouse is so much more sensitive than the human. For example, with radiation exposure the human is more sensitive and there you don't have to worry about delivery of an agent. The radiation gets there. So it certainly is a factor. The type of toxic effect that I've been considering is an effect on the testis. The testis would be the target site and it wouldn't be something that would appear in the semen. So, to do human/animal comparisons would require testicular biopsy which is impractical. It could be done in comparing and understanding the differences between different animal species. For example, different animals exposed to the same dose of chemotherapeutic drug might show different levels of testicular response. One of the factors may be the dose of the drug at the target organs. I think studies like that are important in order to understand the reasons for this and then maybe to be better able to classify compounds in terms of which pattern of extrapolation factor they might fit into. I think we still need the extrapolation factors based on exposure doses. If the toxin is present in the semen, then we have a chance of measuring it in the human semen as well as in the mouse or rat seminal vesicle. It really depends on several things. It depends on the target, if toxin is present in the semen because very little testicular fluid gets out of the semen except for secretions of Sertoli cells.

Dr. Mattison: I'd also like to address that particular issue because it's one that we have been very interested in. We have been looking at compounds that destroy oocytes and we have found enormous strain and species differences in response of the ovary to these particular compounds. One of the things that has been most striking is that if we do intraovarian injections, many of the strain and species differences disappear. And I guess it's not surprising that if we are looking at a pharmacodynamic effect we would expect that to be preserved across a wide species variation. We expect to see change in the pharmacokinetic parameters, and a part of being able to go from an animal model to extrapolation of human risk really does require some measure of the pharmacokinetic parameters and how they vary in terms of delivered dose to the gonad, be it the testis or the ovary.

Dr. Klemme (NIOSH): In context of field studies where there is great dependence on history from the individual participant and where specimens are obtained under somewhat abnormal circumstances, what data are there that relate to the variability in counts and other parameters based on period of abstinence? Secondly, is there reason to consider circadian rhythmicity as a factor affecting what we measure?

Dr. Overstreet: The data available on the effect of abstinence on the semen sperm count suggest that over a period of two or three days the individual is able to replenish his extragonadal sperm reserves. This is in a human. And this is the reason

that we ask for approximately two days of sexual abstinence. Less than that would perhaps be a reflection of sperm reserves that would not be comparable. The second part of your question concerned individuals who are assessed repeatedly over a period of time. We have looked at this in normal fertile men. The variation in sperm counts is quite high with a coefficient of variation of 50% or greater. The variation in the other parameters, motility and morphology, is substantially less than 20%, approaching 15%. So again this is the reason that those parameters, if they can be measured objectively, are more likely to be reliable.

Dr. Meistrich: I would like to make two additional comments. One is that I agree that sperm count as reflected by sperm concentration is dependent on the period of abstinence, but what is more apparent is that total sperm per ejaculate decreases with decreasing period of abstinence. Also, semen volume decreases. This tends to partially cancel making the sperm concentration a little bit more constant parameter than total sperm per ejaculate. If you have a choice between using those indices, sperm concentration is somewhat less dependent on period of abstinence, but I agree with Dr. Overstreet in that it is certainly more dependent on periods of abstinence than the sperm qualities, like morphology, morphometry, and motility. The other point that you asked about was circadian rhythm. There is a recent paper about circannual rhythm where there has been enough data collected to detect a significant circannual rhythm. However, the variation was of the order of 25% over the year with, I think, a decline in late summer. I think that that variation is really small compared to individual variation and although it was picked up with a large population it's smaller than other variability factors.

Dr. Newton (University of California, Irvine) Dr. Mattison, you made a comment in your presentation that beryllium-induced pulmonary function changes had a negative impact on reproduction or fertility, I don't remember which. Does that involve the hormonal or non-pulmonary functions of the lung and what's the mechanism of that?

Dr. Mattison: Actually it was survival of pregnancy. Women who had beryllium lung disease had an enormous mortality in pregnancy as a result of being unable to alter normal respiratory patterns which occur in pregnancy. So it wasn't the hormonal effect, it was an effect on the normal physiological response to pregnancy in humans. Now this exposure is unlikely to occur at the present time. This is old data and in fact, I don't remember it exactly but the mortality rate was something like 75 or 80% in the woman with beryllium lung disease during pregnancy. So that the impact of that occupational lung disease on ability to respond to pregnancy was actually quite large.

Col. Hawkins (Chemical Research & Development Center): My question is for Dr. Meistrich with regard to your last slide on the glycol ethers. It said that the extrapolation for permissible levels was 5 parts per million. I thought that I had read recently that it was being recommended that that level be lowered to approximately 2 ppm. Do you know if your slide represents the official permissible level or is that still in contention at the present time?

Dr. Meistrich: The slide does not represent the official permissible level. I am sorry if I created that impression. It's based on this calculation which is really quite new and actually was submitted in an EPA report. It may or may not be related to what they consider a permissible level. But what I did base the calculation on is just the totally arbitrary assumption of a 1% increase in infertility in the population. What dose of exposure that would correspond to is based on that model. I think it's solely the decision of regulatory agencies or people concerned with risk benefit or a political decision what, if any, increase in infertility is acceptable. I think also what this model showed is that with sperm count reduction there is no threshold. Even at a 10% reduction in sperm count there would be a slight increase in infertility in the human population. It was just a number based on the model with a given arbitrary input of 1% increase in infertility. So, I think this model can be used by regulatory agencies or other bodies to then make their decision.

Col. Hawkins: Yes, I think that's correct. Previously it was, I believe around 5 parts per million but at the present I understand there are recommendations to lower that level in view of either recent data or a relook at the situation and I just wondered about your slide. Thank you.

Dr. Zenick (University of Cincinnati): Just to add a comment to that. At a recent conference on glycol ether that was sponsored by NIOSH the concern voiced there did not appear to be about inhalation exposures at the manufacturing end of production of the methoxy or ethoxy glycol ether. The concern there was in the application of agents that contain these glycol ethers that are essentially uncontrolled and also include extremely high potential for dermal exposure in addition to inhalation exposure. So that when we talk about regulating it down to 2 ppm in those industries that are producing it we are probably still missing the population that's at risk.

Dr. Mattison: I'd like to raise a question that was introduced here earlier and that has come up in previous meetings like this that I have attended. It relates not to scientific issues but to the policy issues. What should the policy of an employer be if there is an occupational exposure which may, for example, effect male reproductive function but for which it appears that there is no effect on female reproductive function? Should that job be classified as a female-only job or, alternatively if there

is an exposure which is thought to be teratogenic, should women be excluded from that job? Or should women be required to be sterilized before they accept that job? That's not a theoretical consideration. At least one company in the United States has allegedly required sterilization of women employees for certain job classifications and it's an extension in a way of the question of how do you evaluate hazard and extrapolate it as risk to humans. I would like to raise it as an issue for discussion because I really don't have a strong feeling one way or the other.

Dr. Zenick: I might make a comment on that. There is a case, I believe it is in Ohio presently, where the employee has sued the firm, he is working in exposures with lead, requesting paternity leave. And the question that was raised was what was the safe level of lead exposure to ensure that he was not at reproductive risk. If his blood leads were above that exposure how long would it take for him to get down to that acceptable level of blood lead and then of course he would then need at least one complete cycle of spermatogenesis to ensure that he had no affected cells or maybe two. So you now are talking about 70 to 100 days in the male and he wants two cycles. Now you are talking about maybe 200 days paternity leave after he gets down to whatever the safe level is, and we couldn't even tell him what the safe level was.

Richard Henderson, Ph.D.: I'll comment on this question of corporate policy. I think you've got to extend that to include fetal toxicity because in some cases one has to be concerned with the developing fetus rather than the adult. My own personal view is that you approach this on a scientific basis. When you have somebody at a significantly higher risk, then you have a responsibility to try and protect that person in one way or another. You have to have a policy that will provide protection. While I have the microphone I'd also like to make one comment and ask for Dr. Meistrich's reaction. He mentioned the fact that one didn't need to be concerned with metabolic processes in regard to the effects of radiation. I'm stretching a little bit what he said but that basically was the way I interpreted it. It's my understanding that radiation has a chance for causing chemical change and, for example, free radical formation and the status of free radical scavengers may be important then in terms of what the reaction is to radiation.

Dr. Meistrich: Yes. I fully agree. There is a difference in cellular sensitivity and the radiation will get to the cell. The status of free radical scavengers, free sulfhydryls, are very important for the sensitivity of a cell. I guess the point that I meant to make is that the radiation will get to the cell. You don't have the additional complicating factors of transport and the pharmacokinetics. You have to worry about the intracellular chemistry as you do with drugs or chemicals in addition to transport. So radiation is a little bit more direct but it's not simple by any means.

Dr. Culver: A comment on the policy issue with regard to males and females. The lead industry, at least that part of the lead industry which manufactures lead acid storage batteries, regularly and routinely excludes females from high lead exposures. The issue has been battled out in the courts and I don't think it's so much of a controversy anymore. It was two or three years ago. As far as I know there is no corporation in the United States that requires that women be sterilized. However, they do exclude women who are capable of reproduction from certain jobs. I'd like to also ask you a question with regard to your statement on nickel carbonyl and its effect on hematopoiesis. Was that an effect on the mother or on the fetus?

Dr. Mattison: It was on the mother. This was work that was done by Sunderman at the University of Connecticut.

Dr. Overstreet: I'd like to take a minute to ask the panel's reaction to a question that hasn't been raised in this forum yet but is often one of interest in discussions of this type. And that is the notion that was fairly popular in the lay press and many of the scientific literatures as well that we should be concerned that male fertility in this country has declined during the past 30 years and that this potentially is a result of environmental factors. I wonder if the panel would care to address the question. Perhaps Dr. Meistrich is most able to do that since he has been looking at the data on the sperm counts which was the basis for some of the concern. Are American men less fertile today than they were in 1940?

Dr. Meistrich: That's a good question. I guess a lot of it is based on that one set of data of Zugman, Steinberger et al. This study of men from fertile couples which were taken pre-vasectomy at a vasectomy clinic showed much lower distribution of sperm counts than most of the other studies such as the controls in the DBCP studies which again are from a small population. Also, a study done in Houston just on selected medical students irrespective of the fertility status. Most studies haven't tested the fertility status of the subjects. Anyway, their sperm counts seemed to conform more to the McCloud and Gold and the French studies. There are data sets that indicate lower sperm counts that seem to be reasonable and some of these data sets seem to be popping up more recently. Then there are other data sets recently that have been obtained that refute that. So I don't think there is any consensus conclusion that there is a reduction in sperm count. There is some indication, but not a firm conclusion.

Dr. Klemme: I'd like to ask Dr. Meistrich to expand or clarify an issue that enters directly into the regulatory question. Among your concluding statements was one that stated that approximately 20% reduction in sperm count would result in roughly a 1% increase in infertility. However, from what I inferred from earlier parts of your presentation, if you looked only at the lower half of the population in terms of sperm count

that 20% reduction would cause a greater than 1% increase in infertility in that lower half of the population in sperm count. If you looked only at the bottom 10% I would guess there would be even a greater increase. If I'm correct in that, what's your suggestion on how to approach that set of issues?

Dr. Meistrich: I haven't analyzed the data that way but just my feeling for the data because the curve was fairly flat at high sperm counts. So it doesn't much matter whether you have a 100 million per ml or 60 million per ml but let's take a more extreme difference, at 100 or 10, it doesn't matter that much. The difference between 10 and 1 is really a big difference and they are both tenfold differences. Certainly an individual with a lower sperm count to start off with is at a greater risk of being pushed from the sub-fertile to the infertile group while someone from the fertile group with the same fractional reduction in sperm count would be pushed into the less fertile group. He may not notice that instead of impregnation in one month it takes him five months and it's not reported as infertility because that's quite normal. Whereas somebody who would have normally conceived within a year and wouldn't have been reported as a case of infertility suddenly doesn't conceive for 5 years with the same proportionate reduction in sperm count now is reported as a case of infertility. So I think your inference is correct that an individual or a group of individuals starting off with a low sperm count is at a greater risk for increase in the incidence of infertility in the population.

Dr. Overstreet: I think this raises an issue as to what information one can give to a worker or to any other individual who is being monitored in a field study as to the implications of the data which may be obtained for that individual. I think we have to be very careful when we talk about group data that describe sperm counts in a group and reductions in sperm counts in a group versus the significance of a reduction in sperm count for an individual. There are some points to make here. One point which I think Dr. Zenick alluded to but which really needs to be emphasized and re-emphasized is that we have no data in our clinical literature which describe any semen parameter for a normal population. The information we have, which Dr. Meistrich showed in his graphs, usually are based on two very highly selected populations a very fertile population and a population attending an infertility clinic. In order to realistically discuss the implications of a semen evaluation for a worker who is not actively trying to conceive a pregnancy, one needs a data base which we do not have. That is, a data base of all normal men regardless of their attempts to achieve a pregnancy. So what we really need to know is what should a sperm count be in a 45 year old worker who is taking a certain number of medications and who has a certain medical history? Then we would be able to tell the man whether in fact his semen parameters are normal in the same way that we would try to do that for any other clinical laboratory test. We don't have the data to do that; therefore, it is not possible to give fertility assessments to individuals

on the basis of results of field studies unless they have no sperm. Now the reason this is true is that we know from other studies that men with very low sperm counts can conceive pregnancies. This comes from studies of contraceptive agents in males which reduce the sperm count very substantially but not completely. Those men may still be fertile. We know from tests of men who have hypogonadal infertility in which replacement therapy with gonadotropins reinitiates spermatogenesis. Sperm concentrations never become very high and in fact they usually stay within what we consider clinically an infertile range. Yet these men are very often fertile. In thinking about sperm counts I also would like you to be aware that many cases of clinical infertility are the basis for many of these data described populations. Those infertility cases are not often a result of numbers. It would be wrong for you to believe that male infertility can be attributed simply to the number of sperm available to get to the egg and reach it. Very often those are testicular dysfunctions that are resulting in sperm dysfunctions which are reflected as a decreased production. But in fact, all the sperm cells being produced may be dysfunctional. So these are the many uncertainties which we have. There is no clear and easy way to tell a man what his particular semen parameters mean unless he has been trying to get his wife pregnant and has not been successful.

Dr. Meistrich: There is another thing I'd like to add. All of our data are statistical. Even if we had the information of what the normal distribution of sperm counts for 45 year old men who have certain medical history would be, all you could tell one is the probability that an individual like him could initiate a pregnancy and also the median time or the average time that it would take someone like him with normal copulatory behavior and a fertile wife to achieve a pregnancy. So even with that information everything still has to be couched in probability terms.

Dr. Mattison: I think that there is something that has to be raised here again and that is that when we assess fertility as opposed to assessing the function of other organs we are assessing the function of organ systems in two individuals. There are a variety of ways that you can look at it. The fertility of a 45 year old man with 10 million sperm per ml who has a 23 year old wife is going to be higher than a 45 year old man with a 49 year old wife. It's not just a function of how his testes are operating. This is an end point that's measured in couples. In a mouse model this has been shown very very elegantly by Generoso who has demonstrated that different strains of female mice can compensate for dominant lethal effects when you treat male animals. So that, for example, some strains of female mice are able to produce normal viable pups after mating with a male given a particular kind of alkylating agent exposure while other strains are unable to produce any pups after treatment of the male. Here is a clear example of the way that the oocyte interacts with what is ostensibly a mutagenized sperm.

Richard Henderson, Ph.D.: I have a vague recollection of reading recently of hormonal effects on exercise and there has been no discussion yet of the effect of exercise on reproduction. I address that to any member of the panel. Have there been animal experiments on exercise versus no exercise? What about exercise in humans?

Dr. Overstreet: It's my impression that most of the studies on exercise physiology with regard to reproduction have been done in females. I know of no studies that have been reported on the male. We have done a very limited number of assessments of long distance runners and we have not been able to see an effect on the semen parameters in those men. Dr. Mattison, you may be able to say something about effects on the female if that's of interest to you.

Dr. Mattison: Yes, there have been studies that have looked at effects of exercise. The major focus of these studies has been effects on the control of the endocrine cycle in women and it's been most strongly noted in competition runners, competition swimmers and ballerinas. There is a series of interesting studies done in New York City looking at professional women in intensely competitive professions such as violinists and ballerinas. It has been suggested that it's the stress of the competition that is altering the integration of the female reproductive system rather than an exertional effect. In fact, among violinists with what was considered to be equivalent levels of psychological stress to those of ballerinas, the menstrual cycle integrity seemed to be maintained. Whereas in the ballerinas the menstrual cycle was quite severely disrupted. The other issue that goes now beyond just normal integrated adult reproductive function is the effect of exertion on pregnancy. We have no data in that area. In a way it's ironic that we are often asked to assess the effect of a chemical exposure on pregnancy when in fact we can't even assess the effect of working on pregnancy.

SESSION IV

TOXICITY OF MIXTURES

Chairman

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PHARMACOKINETIC INTERACTIONS OF MIXTURES

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INTRODUCTION

Most commercial solvents are complex mixtures containing a variety of volatile liquids. In the occupational environment the primary hazard associated with use of solvents is vapor inhalation. The pharmacokinetics of inhaled vapors and gases have been intensively studied and are particularly well-characterized. Early work on inhalation pharmacokinetics focused on the behavior of anesthetic gases and vapors which were considered to be un-metabolized (Haggard, 1924; Kety, 1951; Riggs, 1963). More recently the same type of physiological approach used successfully with anesthetics has been systematically applied by inhalation toxicologists to study the pharmacokinetic behavior of solvent vapors which are readily metabolized in the body (Fiserova-Bergerova et al., 1974; Ramsey and Andersen, 1984). These studies have clearly delineated the importance of biochemical factors (V_{max} and K_m of the metabolizing enzymes), physiological factors (blood flows, ventilation, organ volumes, etc.), and physico-chemical constants (blood:air and tissue:blood partition coefficients) in determining the pharmacokinetic behavior of inhaled vapors. Very little attention, however, has been given to the problems of the influence of co-exposure to other vapors on the pharmacokinetic behavior of an inhaled substance. In those cases where the kinetics of inhaled vapors have been analyzed both singly and in combination with other inhaled substances, the data analysis has usually been qualitative with little effort to quantitate the interactions by use of detailed physiological models.

This paper is divided into two sections. First, we describe several examples from the literature which indicate toxicologic or pharmacokinetic interactions arising during co-exposure to two vapors. Secondly, we discuss the metabolic basis of these interactions and try to generalize these results to illuminate the interactions that are expected during exposure to more complex mixtures. Where possible, the examples in the second portion are given a quantitative basis by applying a physiological pharmacokinetic model to describe these interactions.

SOME EXAMPLES FROM THE LITERATURE

We have selected four examples that describe an effect of co-exposure to a second vapor on either the toxicity or the pharmacokinetics of an inhaled gas or vapor. The first two examples describe toxicologic interactions and the last two describe kinetic interactions. No attempt was made to do a detailed literature search to compile a list of known interactions. Instead, these four examples were selected because at least one of the chemicals in each pair is either of current interest in our laboratory or has been previously studied by our laboratory staff.

Toxicologic Interactions. The chlorinated ethylenes are industrially important chemicals and have very interesting toxicologic properties which vary considerably among the six chemicals in this family. Vinylidene chloride (1,1-dichloroethylene, 1,1-DCE) has the most significant acute toxicity following single inhalation exposures. In fasted male rats the 4 hr LC₅₀ was estimated to be 600 ppm (Jaeger et al., 1974). Vinyl chloride (monochloroethylene, VCM) is not acutely hepatotoxic, but is carcinogenic causing hemangiosarcoma in various experimental animals and in man after repeated inhalation exposures. Both the acute effects of 1,1-DCE and the carcinogenic effects of VCM are associated with epoxide metabolites of the chlorinated ethylenes. Jaeger et al. (1975) determined the acute toxicity of inhaled 1,1-DCE alone and in combination with VCM (Table 1). Co-exposure to 1000 ppm VCM protected fasted, male rats from the hepatotoxic effects of a single exposure to 200 ppm 1,1-DCE. This protection is probably related to competition between the two chemicals for the same active site on the metabolizing enzyme. This competition reduces the amount of 1,1-DCE metabolized to the toxic intermediate in the mixed exposure.

TABLE 1. HEPATOXICITY OF 1,1-DICHLOROETHYLENE IN FASTED MALE RATS

<u>Exposure Group</u>	<u>Serum Transaminase</u>
Air Control	0.20 - 0.40
1,1-DCE (200 ppm)	16.00 ± 8.95
VCM (1000 ppm)	0.24 ± 0.01
1,1-DCE + VCM	0.16 ± 0.02

From Jaeger et al. (1975), Arch. Environ. Health, 30:26-31.

Exposures were for 4 hr and rats were killed 24 hr after the end of exposure. 1,1-DCE is 1,1-dichloroethylene; VCM is vinyl chloride. The activity is serum alanine- α -ketoglutarate transaminase and units are mg pyruvate/ml/hr.

The second example comes from a study of the combined effects of n-hexane and toluene on nerve conduction velocity (NCV) in exposed rats (Takeuchi et al., 1981). Rats were exposed to either 1000 ppm hexane or a combination of 1000 ppm hexane and 1000 ppm toluene for 12 hours/day for 16 weeks. NCV measurements were made at week 0, 4, 8, 12, 16, and 4 weeks after cessation of the daily exposure regimen (Figure 1). While the hexane exposure caused a significant decrease in NCV, the NCV was unchanged in rats exposed to the combination of hexane and toluene. The authors speculate that toluene, a substrate for microsomal oxidation, inhibits the conversion of n-hexane to neurotoxic metabolites, presumably 2,5-hexanedione.

Pharmacokinetic Interactions. These two kinetic examples also relate to inhibitory effects of vapors that are metabolized by microsomal oxidation. Sato and Nakajima (1979) studied the metabolism of benzene and toluene in vitro and determined the time course of toluene in blood of rats given toluene alone or toluene in combination with an equimolar dose of benzene (Figure 2). There was an inhibition of toluene elimination at a benzene dose of 5 mmole/kg but not at doses of 1.25 mmole/kg or less. With reduction in toluene clearance (due to decreased metabolism), there is an increase in area under the toluene blood curve. Thus, overall toluene exposure is increased when metabolic clearance is diminished by co-administration of benzene.

The last, brief example is the effect of carbon tetrachloride on the metabolism of 1,1-DCE. In previously published studies, Andersen et al. (1979) described a closed chamber, recirculated atmosphere system for determining metabolism of inhaled vapors and used it extensively to study 1,1-DCE metabolism. The decline of chamber concentration with time is primarily due to metabolism of 1,1-DCE. If there were no metabolism, there would be uptake until an equilibrium was reached between the rat and the atmosphere and then no further decrease in chamber concentration would occur. The chamber concentration versus time curve with control rats (Figure 3) shows the rapid, continuing metabolism of 1,1-DCE. When the chamber atmosphere also contained 2000 ppm CCl_4 , the metabolism of 1,1-DCE was abolished. As with the other three examples, co-exposure reduced the metabolism of the test vapor.

PHARMACOKINETICS AND ENZYME ACTIVITY

Inhaled vapors are eliminated by two major processes, exhalation and metabolism. Exhalation is first order with respect to mixed venous concentrations of the vapor and should not be significantly affected by the presence of other vapors in the blood unless these vapors have some noxious effect on the lungs. Metabolism is usually capacity-limited with respect to arterial or organ concentration of the vapor and will be prone to competitive inhibition when various components of the mixture all share a common enzymatic active site. Metabolism then is the

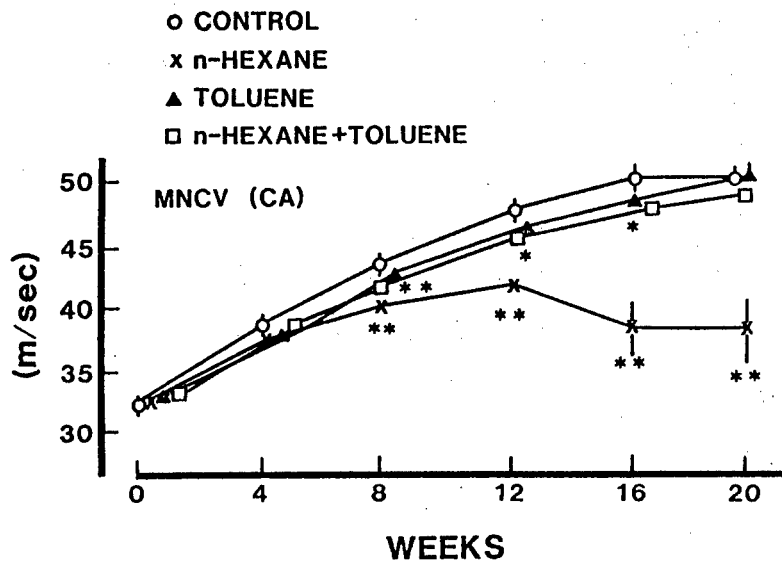


Figure 1. Mean whole tail nerve conduction velocity (NCV) in male rats exposed to hexane, toluene, or a combination. Exposure concentrations were 1000 ppm for 16 weeks followed by a 4 week recovery period. The CA designation is for the placement of electrodes for measuring whole tail NCV. The figure is reproduced from Takeuchi et al. (1981) with permission.

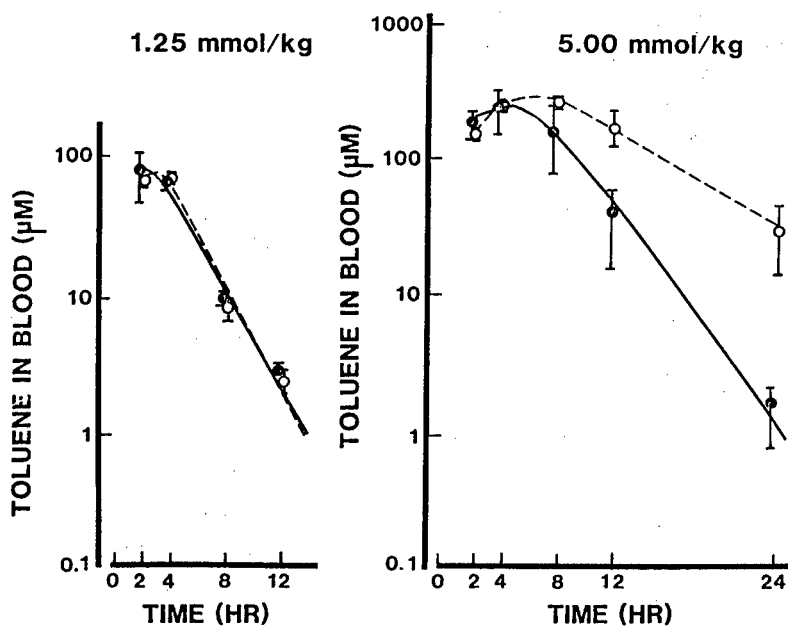


Figure 2. Time course of toluene in blood after intraperitoneal injection of equimolar amounts of toluene and benzene (○) or after injection of toluene alone (○). Reproduced from Sato and Nakajima (1979) with permission. The presence of benzene decreases toluene clearance at the higher but not at the lower concentration.

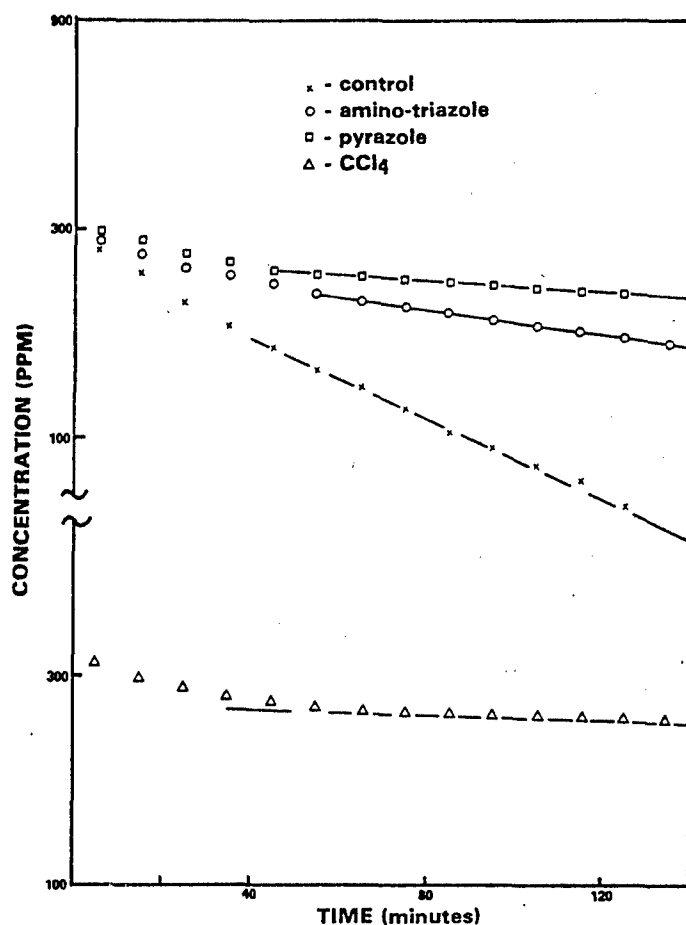


Figure 3. Gas uptake studies with 1,1-dichloroethylene (1,1-DCE). The rapid rate of disappearance of 1,1-DCE in control rats (9 rats in a 31 L chamber) is due to metabolic utilization of 1,1-DCE (x). When CCl₄ is added to the chamber (Δ) the rate of uptake of 1,1-DCE is very greatly reduced.

process most likely to be involved in the altered kinetics of a chemical in a mixture compared to the kinetics of the chemical when delivered alone. Metabolism may either be increased by induction (although none of the examples dealt with this possible behavior) or be decreased by competition for available enzyme or by destruction of metabolizing enzyme by some toxic effect of one of the components in the mixture.

EFFECTS OF INDUCTION

We can distinguish several types of toxicity associated with vapor inhalation. The parent chemical may be responsible for toxicity or, as is more often true with solvent chemicals, a reactive metabolite may be the toxic agent. In the first instance, we expect toxicity to be related to integrated target tissue dose which is usually closely related to area under the blood curve. In the second case, internal dose is more likely to be related to some measure of the amount metabolized. For an enzymatic, capacity-limited process, total amount metabolized will not be a simple function of inhaled concentration. Nonetheless, enzyme induction can have an impact on toxicity whether the inhaled vapor itself or a metabolite is the active toxic species.

Andersen et al. (1984) have recently studied the induction of styrene metabolizing capacity in rats. Styrene is an example of a soluble vapor and has a blood:air partition coefficient of 40. At low inhaled concentrations, styrene is very readily metabolized and virtually all styrene reaching the metabolizing organ is removed from the blood. Under these conditions a steady-state is achieved where the blood:air concentration ratio is only about 2 or 3. At higher exposure concentrations where metabolism is saturated, the steady-state concentration ratio will more nearly approach the blood:air partition coefficient.

The phenomenon of concentration-dependent partitioning has been used to determine the metabolism of styrene under various conditions. The partitioning curves for styrene in naive and induced rats (both styrene-induced and phenobarbital-induced rats) are complex as shown in Figure 4. Pyrazole inhibits styrene metabolism and shifts the partitioning curve in a direction opposite to that seen on induction. For a soluble vapor like styrene, then, altering enzyme activity has a dramatic effect on circulating blood levels. A good example of this is provided by observing blood styrene concentrations during a 48 hr continuous exposure of rats to 400 ppm styrene (Figure 5). Here blood levels fall to a sixth of their maximum level as exposure proceeds beyond 12 hr. The threshold concentration for induction by continuous exposures is somewhere between 200 and 400 ppm, probably closer to 200 ppm (Andersen et al., 1984).

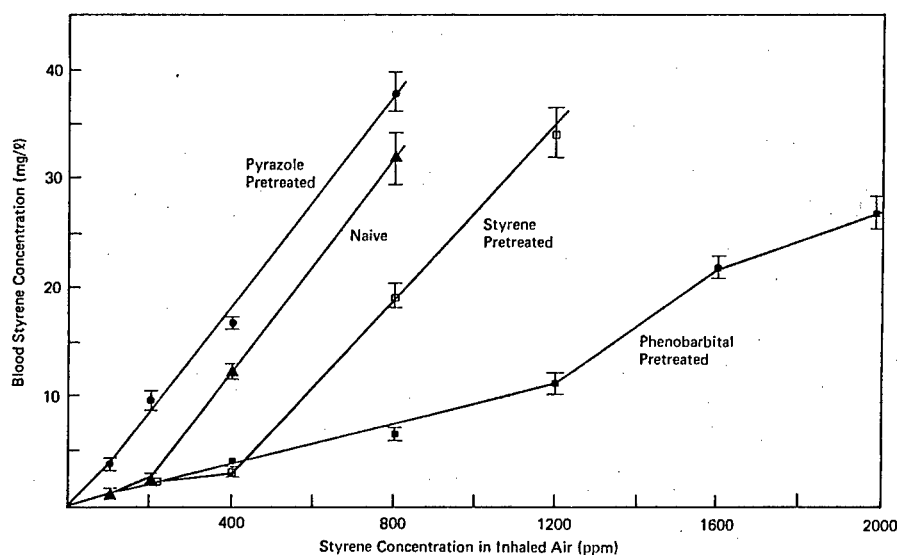


Figure 4. Blood:air partitioning of styrene in male Fischer rats. Rats were exposed to styrene for 6 hr and then killed. Blood was taken from the abdominal aorta for styrene analysis. The plot is the average blood concentration in groups of 3-6 rats at particular exposure concentrations of styrene (x-axis). Pyrazole inhibits metabolism

and shifts the curve to the left. Prior styrene exposure (1000 ppm, 6 hr/day, the 4 days before testing) or pretreatment with phenobarbital (80 mg/kg on each of the 4 days before styrene exposure) induced metabolism and shifts the curves to the right.

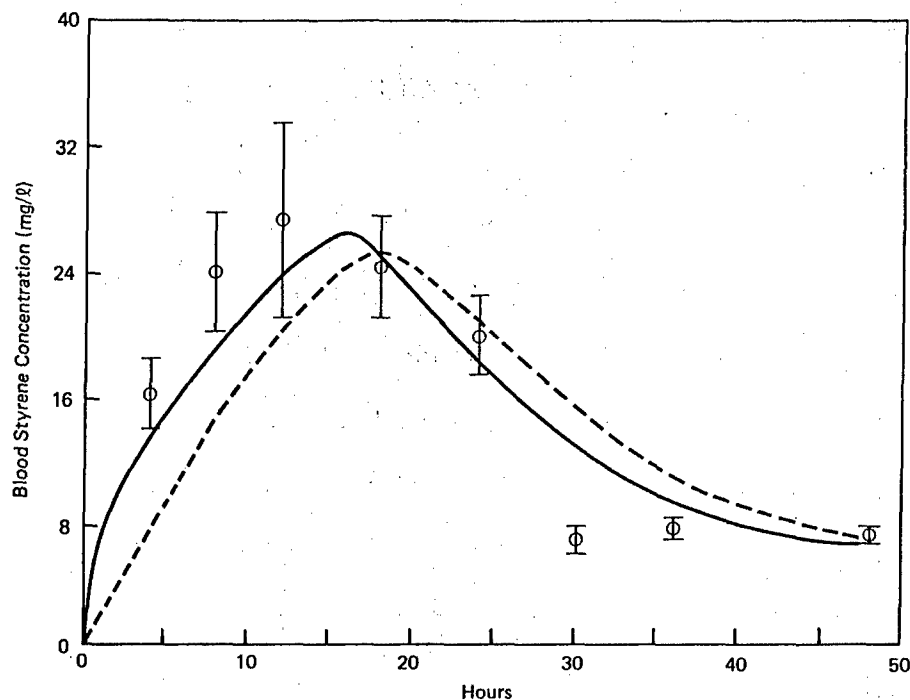


Figure 5. Blood levels of styrene during continuous, 48 hr exposure of male Fischer rats to 400 ppm styrene. Groups of rats were exposed for various times, then removed from the chamber and killed. Blood was drawn from the abdominal aorta for measuring styrene. The decreasing blood concentrations after 15 hr reflect induction of styrene metabolizing enzyme activity by this continuous exposure. (See Andersen et al. (1984) for a full description of the time- and concentration-dependencies of this induction process.)

INHIBITION

Induction increases metabolite production and lowers steady-state blood concentrations of parent vapor. However, induction is probably of less practical significance than enzyme inhibition. All four of the examples used appear to illustrate competitive relationships between substrates metabolized by the same enzyme system. One unanswered question in assessing potential inhibitory interactions of vapors is the extent to which the metabolism of structurally diverse xenobiotic vapors is interdependent. The microsomal oxidase system has broad substrate reactivity and it is possible that the metabolism of many diverse vapors will show some inhibitory interactions. With soluble vapors, inhibition can greatly increase circulating blood concentrations. This was evident in the earlier figure showing the effect of pyrazole on styrene partitioning. This behavior will lead to enhanced toxicity if the parent chemical is the active toxic moiety.

PHARMACOKINETICS OF INHALED HEXANE

In our laboratory we have recently studied the pharmacokinetics of inhaled hexane. Part of this work is being done collaboratively with scientists at the Chemical Industry Institute of Toxicology (CIIT) and part of it is an in-house project. We were led to a study of hexane because it is found in a variety of hydrocarbon distillate fuels of AF interest and, along with benzene, is one of the few components in these mixtures with established toxic effects.

Hexane is metabolized sequentially to mono- and then dioxygenated metabolites. The primary forms of these intermediates are methyl-n-butyl ketone and 2,5-hexanedione. Both the pathway which converts hexane to methyl-n-butyl ketone and that converting methyl-n-butyl ketone to the dione involve ω -1 oxidation catalyzed by microsomal monooxygenase enzymes. Work from CIIT by Baker and Rickert (1981) showed that blood 2,5-HD levels during hexane exposure actually decreased as the inhaled hexane concentration was raised above 1000 ppm. In addition, maximal blood concentrations of 2,5-HD occurred at some time after the end of exposure (Table 2). The data suggested some inhibitory interaction in the hexane metabolic pathway, specifically an interaction of hexane on further metabolism of the monooxygenated intermediates. However, this notion was not tested by quantitative modeling of the blood level data.

TABLE 2. BLOOD CONCENTRATION OF HEXANE AND ITS METABOLITES IN MALE RATS EXPOSED TO HEXANE FOR 6 HOURS

Inhaled Hexane ppm	Hexanedione (mg/L)		2-Hexanone (mg/L)	Hexane (mg/L)
	(0.5 Hr)	Peak (Time)		
500	3.6	3.6 (0.5)	0.7	1.3
1000	5.6	6.1 (2.0)	1.9	2.2
3000	2.8	6.7 (1.0)	4.2	8.4
10000	1.2	3.8 (6.0)	8.0	20.9

Exposures were with Fischer 344 rats. With hexane and 2-hexanone blood concentrations are at the end of the 6 hr exposure. With 2,5-hexanedione the times refer to hrs after exposure and peak is the highest observed dione concentration. These data are either directly from Baker and Rickert, Toxicol. Appl. Pharmacol., 61:414-422 (1981) or were graciously supplied for use in this paper by Dr. Douglas Rickert, Chemical Industry Institute of Toxicology.

In our laboratory we have examined the metabolism of hexane by gas uptake methods and attempted to provide a coherent, comprehensive description of hexane metabolism in the rat. This description includes inhibitory interactions in the two microsomal oxidation reactions. This work from our AF laboratory on

hexane metabolism illuminates the kinetic interactions between a hydrocarbon and ketone and constitutes the final topic of this paper.

METABOLISM OF INHALED HEXANE

The results of gas uptake studies with hexane are shown in the next figure (Figure 6). Hexane has a low blood:air partition coefficient and a minimal loss rate from an empty chamber. All the loss after about 40 minutes is associated with hexane metabolism in the rats. Consistent with results with inhaled pentane (Filser et al., 1983), we found that hexane was metabolized by both a first-order and a saturable pathway. The downward curvature with the naive rat represents the transition from saturation to first-order conditions for the capacity-limited metabolic pathway. Hexane metabolism was partially inhibited by simultaneous exposure to toluene or by pretreatment with pyrazole (Figure 6).

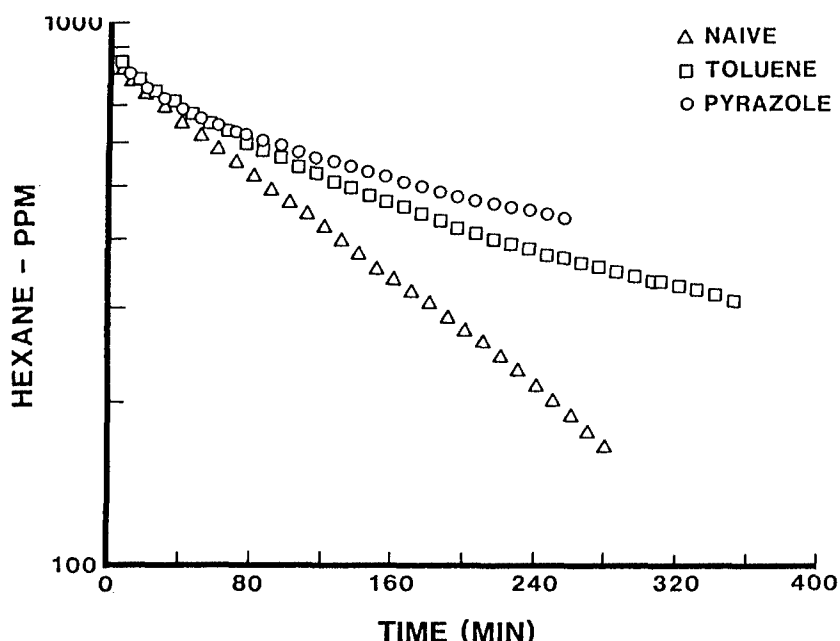


Figure 6. Gas uptake studies with n-hexane in male Fischer rats. Three rats were placed in a 10 L desiccator jar chamber and sufficient n-hexane introduced to give an initial chamber concentration of 1000 ppm. These curves show chamber disappearance of hexane for naive rats, for rats pretreated with 320 mg pyrazole/kg (to inhibit microsomal oxidation), or for rats simultaneously exposed to hexane and 1000 ppm toluene. Both pyrazole and toluene decrease uptake indicating inhibition of total hexane metabolism.

Hexane metabolism was also inhibited by pretreatment with either methyl-n-butyl ketone or 2,5-hexanedione (Figure 7). There were significant differences though in the nature of the

interactions. Based upon a pharmacokinetic evaluation using a physiological model, methyl-n-butyl ketone was presumed to inhibit the saturable pathway of hexane metabolism. Hexanediol had no effect on hexane metabolism initially, but produced significant inhibition at later times. This behavior suggested that the 2,5-HD inhibits methyl-n-butylketone metabolism and as the methyl-n-butylketone accumulates, hexane metabolism is diminished.

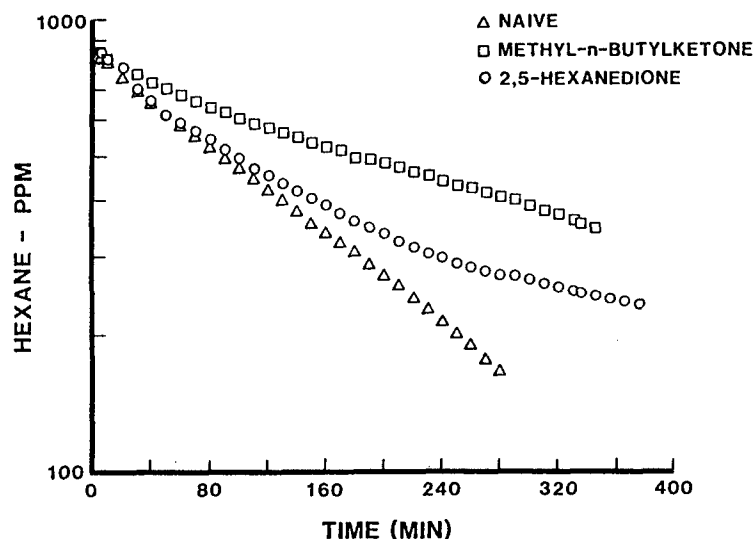


Figure 7. Gas uptake studies with n-hexane in male Fischer rats pretreated with various metabolites of n-hexane. In these experiments rats were given 406 mg methyl-n-butyl ketone/kg or 487 mg 2,5-hexanediol/kg 0.5 hr before being placed in a gas uptake chamber. Both pretreatments decrease hexane uptake as compared to controls, but the inhibition caused by the two compounds is qualitatively different.

A simplified outline of hexane metabolism is shown in Figure 8. We modeled this system with inhibitory interactions on step 1 by methyl-n-butylketone and on step 2 (presumably the same

SCHEME FOR HEXANE METABOLISM USED FOR PHARMACOKINETIC MODELING

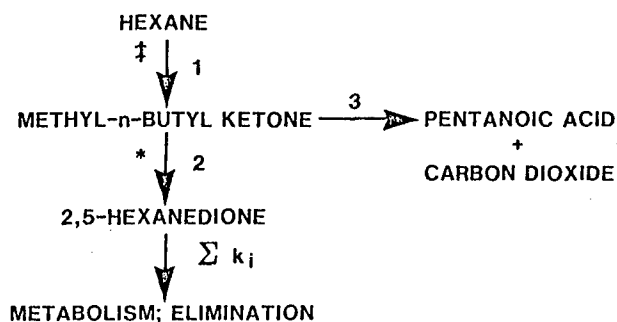


Figure 8. General pathway of n-hexane metabolism.

* HEXANE COMPETITIVELY INHIBITS STEP 2.

‡ METHYL-n-BUTYL KETONE COMPETITIVELY INHIBITS 1.

enzyme) by hexane. Figure 9 demonstrates the model predictions for high concentrations and compares them to observed kinetic data obtained by scientists at CIIT (Figure 9). The description used assumed that hexane was a much more effective inhibitor of step 2 than methyl-n-butylketone was of step 1. An interesting aspect of behavior is seen in the postexposure period where hexane concentrations fall very rapidly while 2,5-hexanedione increases. This occurs because hexane is very poorly soluble and is rapidly eliminated by exhalation. The hexane inhibition on step 2 is released very suddenly on cessation of exposure and there is an increase in flux from the mono- to diketone. This leads to maximal metabolite concentration in the postexposure period.

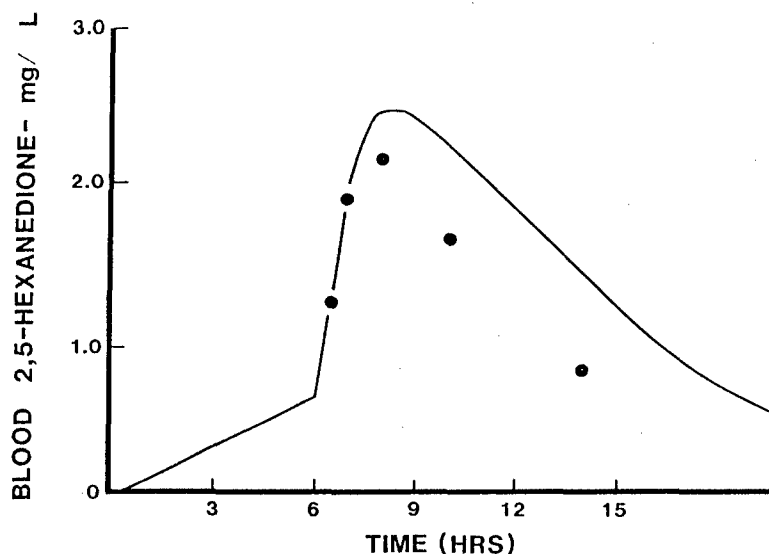


Figure 9. Time course of blood 2,5-hexanedione during and after exposure to 10,000 ppm n-hexane for 6 hours. Data were kindly supplied by D. Rickert and are derived from experiments described in Baker and Rickert (1981). The smooth curve was calculated with the competitive inhibition model described in the test.

Enhanced metabolism at the end of exposure is not expected to be unique to the hexane:methyl-n-butylketone pair. Co-exposure to metabolized vapors of very different solubilities should yield similar behavior. For instance, similar behavior should occur on exposure to 10,000 ppm hexane and 100 ppm of a soluble vapor whose metabolism is inhibited by hexane. From example 2 above (Takeuchi et al., 1981), a probable candidate would be toluene. The hexane should inhibit toluene metabolism during exposure but be rapidly cleared by exhalation at the cessation of exposure. Toluene concentration will be enhanced

during the co-exposure due to inhibition of metabolism. After exposure, metabolic clearance of toluene will increase and give the greatest metabolite burden (as instantaneous rate) in the early postexposure period. This behavior is complexly related to competitive metabolism and relative solubilities of the inhaled vapors. Nonetheless, it is important to bear in mind that the temporal relationship of toxicity may also be shifted during exposure to multiple, chemically-diverse solvent chemicals.

SUMMARY

Elimination of inhaled vapors takes place via exhalation and metabolism. Exhalation is a first-order process at all concentrations and not likely to be affected by other dissolved vapors. Metabolism is often capacity-limited and a frequent site of competitive interactions even with vapors which are not close structural analogs. Manipulation of enzyme activities either through induction or inhibition will alter both rates of metabolism and circulating blood concentrations of inhaled vapors. Co-exposure to mixed atmospheres will likely lead to competitive, inhibitory interactions in the metabolic pathways. Very complex interactions can occur when vapors are biotransformed by multiple reactions each requiring the same enzyme activity. An example of this behavior is seen in the pharmacokinetic analysis of the metabolism of inhaled n-hexane. Complete kinetic analysis of the n-hexane methyl-n-butyl ketone pair requires more detailed knowledge of competitive interactions than is presently available, but a consistent picture has emerged by use of a relatively simple kinetic approach. An appreciation of the altered pharmacokinetics expected during co-exposure to other vapors can be useful in guiding interpretation of observed toxic effects and in formulating coherent, pharmacokinetic models to provide a quantitative framework for assessing chemical interactions in complex atmospheres.

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THE TOXICITY OF COMPLEX MIXTURES

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INTRODUCTION

In discussing the topic of the toxicity of complex mixtures, a definition of what we mean by "complex" is required. I shall consider any mixture of toxicants containing two or more components as complex. Statisticians have proposed various mathematical models to describe a number of types of toxicological action that mixtures may exhibit and I shall touch on these briefly. In addition, I will outline some studies on two component air pollutant mixtures and also another study with a mixture containing several hundred components to show how toxicologists, in fact, deal with these problems and to what extent they refer to these mathematical models in current practice.

JOINT ACTION STATISTICS

Bliss, in 1939, was one of the first statisticians to propose a classification of what is called "joint action", which was limited to quantal response phenomena (Bliss, 1939). Further developments were made by Finney (1942), but the most advanced treatment of the subject is associated with Hewlett and Plackett (Plackett and Hewlett, 1952; Hewlett and Plackett, 1959). An up-to-date discussion can be found in the 1977 reprint of Finney's text on Probit Analysis (1977).

The kinds of joint action which Plackett and Hewlett recognized in 1952 are shown on Table 1. Note that, with both similar and dissimilar joint action, the possibility of interaction is allowed for, which may be either a synergism or antagonism. Because of the great theoretical, and practical, significance of interactions, the possibility always excites much interest among investigators. However, it should be remembered that such interactions appear to be of infrequent occurrence. This is recognized by the ACGIH in the section on threshold limit values for mixtures in the TLV booklet. Their remarks are shown on Table 2. Note that these comments refer to cases of similar joint action. They do, however, go on to consider briefly the possibility of independent dissimilar action. The use of these TLV mixture guidelines has been found in practice to be useful, and few surprises have been encountered.

TABLE 1. TYPES OF JOINT ACTION

Similar joint action:	
simple similar action	- interaction absent
complex similar action	- interaction present
Dissimilar joint action:	
independent action	- interaction absent
dependent action	- interaction present
Similar joint action:	Two positions, separately or jointly, cause the same physiological system to react.
Dissimilar joint action:	Two positions, separately or jointly, cause different physiological systems to react.
Interaction:	The presence of toxicant A influences the amount of toxicant B reaching B's site of action or the changes produced by B at B's site of action.

TABLE 2. THRESHOLD LIMIT VALUES FOR MIXTURES

"WHEN TWO OR MORE HAZARDOUS SUBSTANCES, WHICH ACT UPON THE SAME ORGAN SYSTEM, ARE PRESENT, THEIR COMBINED EFFECT, RATHER THAN THAT OF EITHER INDIVIDUALLY, SHOULD BE GIVEN PRIMARY CONSIDERATION. IN THE ABSENCE OF INFORMATION TO THE CONTRARY, THE EFFECTS OF THE DIFFERENT HAZARDS SHOULD BE CONSIDERED AS ADDITIVE."

While mathematical models are available to test for the various types of joint action, the computations become formidable if there are more than three or four components in the mixture. It should be remembered that it is not possible to predict the type of joint action that may be exhibited by a mixture, even though the regression lines of the responses of the individual components are known. One can test the observed responses to the mixture in the various mathematical models and then make a statement of this kind: "The mixture behaved in accordance with the mathematics of such-and-such a type of joint action." This does not necessarily mean that, at the mechanistic level, the mixture did, in fact, act that way.

Most of the development of these models has derived from studies such as the effect of derris root on aphids or digitalis on frogs, and not on mammalian assays with which we are more familiar. My impression is that toxicologists should design their studies so that the results are susceptible of analysis by the statisticians' joint action models and this might enable statisticians to effect further refinements in the models. In any case, although pharmacologists utilize these designs, little use seems to have been made of the models by mammalian toxicologists. We shall see how, in fact, the toxicologist usually proceeds by considering two examples of joint action investigations.

AIR POLLUTANT MIXTURES

In a series of studies conducted some years ago, various common air pollutants were added to clean air either singly or in binary mixtures. In one study, rats were exposed for one year to lead chlorobromide, which is the predominant lead salt coming from the exhaust of automobile engines burning leaded gasoline. Other exposed groups received the lead compound in combination with nitrogen dioxide, or sulfur dioxide, or carbon monoxide. Measurements of blood lead levels were made at termination. The results are shown in Table 3.

TABLE 3. BLOOD LEAD LEVELS
(micrograms Pb/100 ml. blood)

	<u>Male</u>	<u>Female</u>
1. Control	26.0	17.9
2. 0.54 mg/L PbClBr	65.2	77.0
3. 0.60 mg/L PbClBr + 0.44 ppm NO ₂	37.8	74.6
4. 0.54 mg/L PbClBr + 9.54 ppm SO ₂	23.2	43.3
5. 0.47 mg/L PbClBr + 66.07 ppm CO	87.9	152.6
1. vs. 2.	S	S
1. vs. 3.	NS	S
1. vs. 4.	NS	S
1. vs. 5.	S	S
2. vs. 3.	S	NS
2. vs. 4.	S	S
2. vs. 5.	NS	S

It will be noted that the blood levels in females were all significantly higher than controls, but in two of the male groups, those simultaneously exposed to nitrogen dioxide and sulfur dioxide, there was no elevation of blood lead concentrations.

Consider also the case of Group 5. Here there was no effect in male rats when carbon monoxide was also present, but there was a significant increase in blood lead levels in females simultaneously exposed to carbon monoxide. Looking only at the males, we might be inclined to think that this was a case of dissimilar joint action with interaction absent, but with the females, some form of interaction appears to have occurred. The simple analysis of variance that revealed the results shown in Table 3 is not adequate to allow analysis in terms of the joint action models listed earlier. This is why I suggested that the toxicologist should work with the statistician so that studies are designed to yield the data needed for joint action analysis.

UNLEADED GASOLINE

Let us now turn to a really complex mixture, automotive gasoline. Gasoline contains well over 500 individual compounds. In a recent study (MacFarland, 1982; MacFarland et al., 1984), rats and mice were exposed for 2 years to the vapor of unleaded gasoline at concentrations of 67, 292 and 2056 ppm. Tables 4 and 5 show the chemical analytical information available on the gasoline used in the study.

It will be seen that, although there were more than 542 possible isomers in the five major classes of hydrocarbons in the gasoline, quantitative compositional results were obtained for only 151 of them. Even this, of course, represents a very costly and lengthy job of chemical analysis.

Let us examine some of the results of this study. Provision was made for interim sacrifices at scheduled time points. At the first of these, at 3 months, renal tubular disease was evident in the male rats. The changes seen have been observed previously by several investigators in studies with various petroleum solvents and hydrocarbons (Carpenter et al., 1975 a,b; 1977; Cockrell et al., 1983; Pitts et al., 1983). However, the striking finding occurred in male rats at termination, after they had undergone exposure for a little over 2 years. A dose-related incidence of renal carcinomas was observed. Without recounting all the speculations to which this finding gave rise, let us consider only one hypothesis that was advanced. Suppose that the terminal carcinomas were induced largely, or entirely, from one class of compounds from the 5 classes present in gasoline. Suppose further that there is a causal link between the early nephrotoxic tubular disease and the carcinomas. If we can accept this as a working hypothesis, then it would be possible to screen individual compounds from the various classes in a short-term assay. If any are indicated, they could be candidates for a confirmative chronic study to see if, indeed, carcinomas appear after 2 years. Such a program is in progress at the moment and there is some evidence to indicate that isoalkanes are the active class.

TABLE 4. DETAILED COMPOSITION OF GASOLINE

Compound Class	Carbon No. Range	No. of Isomers		Volume % in Fuel	No. of Major Contributors	Accounting For	
		Possible	Analyzed For				
Alkanes	C ₃ thru C ₁₀	8	8	11.40	3	10.19	90%
Isoalkanes	C ₄	1	1	1.14	1	1.14	
	C ₅	2	2	10.26	1	10.26	
	C ₆	4	4	8.99	3	8.81	
	C ₇	8	8	4.77	4	4.54	
	C ₈	17	14	16.73	4	11.75	
	C ₉	34	22	2.01	4	1.51	
	C ₁₀ thru C ₁₃	>75	-	2.65	no information		
Total Isoalkanes	C ₄ thru C ₁₃	>141	51	46.55	17	38.01	82%
Cycloalkanes	C ₅	1	1	0.15	1	0.15	
	C ₆	2	2	1.05	1	0.97	
	C ₇	7	7	1.09	3	0.77	
	C ₈	23	16	0.74	-	-	
	C ₉	76	23	1.03	-	-	
	C ₁₀ thru C ₁₃	>76	-	0.62	no information		
Total Cycloalkanes	C ₅ thru C ₁₃	>185	49	4.68	5	1.89	40%
Alkenes	C ₂	1	1	0.00			
	C ₃	1	1	0.03	1	0.03	
	C ₄	4	4	0.90	2	0.75	
	C ₅	6	6	1.29	3	1.22	
	C ₆	17	17	1.40	2	1.26	
	C ₇ thru C ₁₂	>128	-	5.34	no information		
Total alkenes	C ₂ thru C ₁₂	>157	29	8.96	8	3.26	36%
Benzene	C ₆	1	1	1.69	1	1.69	
Alkylbenzenes	C ₇	1	1	3.99	1	3.99	
	C ₈	4	4	9.83	4	9.83	
	C ₉	8	8	7.73	3	5.33	
	C ₁₀	22	-	2.11	no information		
	C ₁₁	>22	-	0.52	no information		
	C ₁₂	>>22	-	0.21	no information		
Total Alkylbenzenes	C ₆ thru C ₁₂	>>36	14	26.08	9	20.84	80%
Indans/Tetralins	C ₉ thru C ₁₃	large	-	1.54	no information		
Naphthalenes	C ₁₀ thru C ₁₂	15	-	0.74	no information		
Total Aromatics	C ₆ thru C ₁₃	>51	14	28.36	9	20.84	73%
<u>Summary</u>							
Alkanes	C ₃ thru C ₁₀	8	8	11.4	3	10.2	90%
Isoalkanes	C ₄ thru C ₁₃	>141	51	46.5	17	38.0	82%
Cycloalkanes	C ₅ thru C ₁₃	>185	49	4.7	5	1.9	40%
Alkenes	C ₂ thru C ₁₂	>157	29	9.0	8	3.3	36%
Aromatics	C ₆ thru C ₁₃	>51	14	28.4	9	20.8	73%
TOTAL		>542	151	100.0	42	74.2	

TABLE 5. IDENTIFICATION OF MAJOR CONTRIBUTORS**Alkanes (3)**

n-butane
n-pentane
n-hexane

Isoalkanes (17)

isobutane	2,2,4-trimethylpentane
isopentane	2,3,4-trimethylpentane
2-methylpentane	2,3,3-trimethylpentane
3-methylpentane	2,2,3-trimethylpentane
2,3-dimethylbutane	2-methyloctane
2-methylhexane	3-methyloctane
3-methylhexane	4-methyloctane
2,3-dimethylpentane	2,2,5-trimethylpentane
2,4-dimethylpentane	

Cycloalkanes (5)

methylcyclohexane	cyclopentane
1,cis, 3-dimethylcyclopentane	methylcyclopentane
1,trans, 3-dimethylcyclopentane	

Alkenes (8)

propylene	trans pentene-2
trans butene-2	cis pentene-2
cis butene-2	2-methylpentene-1
pentene-1	2-methylpentene-2

Aromatics (9)

benzene	p-xylene
toluene	1-methyl, 3-ethylbenzene
ethylbenzene	1-methyl, 4-ethylbenzene
o-xylene	1,2,4-trimethylbenzene
m-xylene	

You will have noted that toxicologists, confronted with the problem of characterizing the toxic effects of complex mixtures, appear to follow fairly obvious lines of investigation and make little use, if any, of the mathematical models of joint action. It is my view that more exploration of the value of these models should be considered when dealing with complex mixtures.

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**TERATOGENICITY STUDIES OF CARBARYL AND
MALATHION ALONE AND IN COMBINATION IN VARIOUS LABORATORY ANIMALS**

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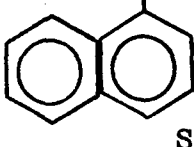
INTRODUCTION

Despite an extensive research effort over the last few decades to understand the toxicity and teratogenicity of carbaryl and malathion, very little has been published on the effect of these compounds in combination or exposure within a short period of time of each other. Recently, much attention has been directed towards the health effects of each of these pesticides individually, as well as in combination. Agricultural use of pesticides carries with it potential hazards to man directly by toxic residues in the food or through exposure in the environment. A synergistic effect may occur through the exposure to different pesticides at the same time. One of the many combinations in use today is carbaryl (1-naphthyl N-methyl carbamate) and malathion (S-(1,2-dicarbethoxyethyl) O,O dimethyl dithiophosphate). This combination is produced by a German company under the name of Super Alexan®. It is effective against house and garden pests. Carbamates and organophosphates exert their primary action on insects, through the inhibition of the cholinesterase enzyme by carbamoylating and phosphorylating, respectively, the esteratic site of the enzyme which causes an abnormal accumulation of acetylcholine in the tissues (Hazleton and Holland, 1953; Carpenter et al., 1961; Murphy, 1975).

Most of the reports deal with the administration of a single compound; thus, only a few investigators have studied this combination. One study looked at the alteration in LD₅₀ of the compounds when used simultaneously (Keplinger and Deichman, 1967). Carbaryl has an LD₅₀ of 850 mg/kg, whereas malathion has an LD₅₀ of 1375 mg/kg in Sherman strain male rats (Gaines, 1960). When the mixtures of carbaryl and malathion were administered to Osborne-Mendel strain male rats in equitoxic doses based on their LD₅₀, Keplinger and Deichman (1967) observed a synergistic response. The expected LD₅₀ was 1.8 times greater than the observed LD₅₀ (Table 1). Therefore, carbaryl and malathion may affect each other in either their mechanism of detoxification or their

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TABLE 1. BASIC INFORMATION ON CARBARYL AND MALATHION

<u>COMPOUND</u>	<u>STRUCTURE</u>	<u>M.W.</u>	<u>LD₅₀(mg/kg)</u>	<u>REFERENCE</u>
Carbaryl	$\text{O}=\text{C}-\text{O}-\text{NH}-\text{CH}_3$  S	201.22	Sherman♂ Rats; 850	Gaines (1960)
Malathion	$(\text{CH}_3\text{O})_2-\text{P}-\text{CHCOOC}_2\text{H}_5$	330.36	Sherman♂ Rats; 1375	Gaines (1960)
Carbaryl/Malathion	$\text{CH}_2\text{COOC}_2\text{H}_5$		Osborne-Mendel♂ Rats Expected: 403 Observed: 221	Keplinger & Deichman (1967)

mechanism of action. The same authors reported that a similar effect was observed when carbaryl and other organophosphates such as parathion and diazinon were administered. It is worth noting that malathion's effect with carbaryl was the greatest compared to the other organophosphates examined. Species differences exist in relation to the LD₅₀ range, since the expected combination LD₅₀ for the Osborne-Mendel strain was 403 mg/kg and for the Sherman strain it would be 1112 mg/kg.

In the area of teratology, little has been studied concerning the interaction of chemicals, especially these two pesticides. This report will discuss in sufficient detail those aspects of teratogenicity and the mechanism which should allow for a better understanding of the toxic actions of these pesticides.

Reports of Carbaryl's Teratogenicity

The effects of carbaryl on various aspects of teratology in laboratory animals have been reported by a number of investigators (Table 2). There has been considerable disagreement among these studies conducted on whether carbaryl affects fetal development. The report of the teratogenic effects of carbaryl on the Beagle dog stimulated many of the following studies. Daily administration of carbaryl in the diet throughout the gestational period produced an increased incidence in malformations. Anomalies present include an opening in the ventral wall, ecaudate pups, visceral agenesis and brachygnathia (Smalley et al., 1968). Doses of 25 and 50 mg/kg carbaryl resulted in 50 percent abnormalities, whereas lower doses of 6.25 and 12.5 mg/kg carbaryl led to approximately 17 percent abnormalities. The same authors reported an increase in resorptions following these doses of carbaryl.

TABLE 2. TERATOLOGY STUDIES WITH CARBARYL

LABORATORY ANIMAL AND REFERENCE	DOSE (MG/KG) AND ROUTE	TREATMENT PERIOD (DAY)	RESPONSE
Beagle dog (Smalley et al., (1968)	6-50; diet	0-62	open ventral wall; ecaudate pups; visceral agenesis; brachygnathia; resorptions
CF-1 Mice (Murray et al., (1979)	150; oral 1166; diet	6-15 6-15	+dam wt. gain +dam wt. gain +fetal size
Dunkin Hartley G.P. (Weil et al., 1973)	200; oral 300; diet	10-24 10-24	+dam wt. gain --- ^a
Coulston G.P. (Robens, 1969)	300; oral capsules	11-20	vertebral malformations
N.Z. Rabbits (Murray et al., 1979)	200; oral	6-18	omphalocele resorptions +dam wt. gain
N.Z. Rabbits (Robens, 1969)	50-200; oral capsules	5-15	--- ^a
Golden Syrian hamsters (Robens, 1969)	250; oral	6-8	fetal mortality
Rhesus monkeys (Dougherty et al., 1971)	20; oral	entire gestation	abortions
Harlan Wistar rat (Weil et al., 1972)	500; diet	0-20	+dam wt. gain
White Leghorn chick embryo (Proctor et al., 1976)	0.1-3 mg/egg; yolk sac injection	4	type I anomalies ^b
White Leghorn chick embryo (Swartz, 1981)	1-10 mg/egg; yolk sac injection	1	embryo mortality subcutaneous edema ^c

^a --- Represents no toxic response was observed

^b Abnormal feathering, micromelia, increased incidence of straight legs and growth retardation

^c The response was observed on days 5 and 12 of incubation.

The type of vehicle used to deliver the carbaryl appears to be an important factor in the anomalies observed as demonstrated in Table 2. Murray et al., (1979) examined carbaryl's teratogenic potential on CF-1 mice both through oral intubation and diet after exposure for days 6-15 of the gestational period. The dietary intake per day was 1166 mg/kg whereas the dose for gavage was 150 mg/kg/day. Even though the oral intubation dose was approximately 1/10 of the dietary dose, both produced a significant decrease in maternal weight gain during the gestational period, but neither dose produced terata (including visceral and skeletal abnormalities). However, only the dietary route produced a significant decrease in fetal size. Other investigators examined the same problem in different species. Dunkin-Hartley guinea pigs were exposed to 50, 100, and 200 mg/kg carbaryl by oral intubation and 100, 200, and 300 mg/kg carbaryl by dietary intake daily during the gestation. A decrease in maternal weight gain was noted for the 200 mg/kg oral intubation dose, whereas doses as high as 300 mg/kg of the diet had no effect (Weil et al., 1973). When Robens (1969) administered 300 mg/kg in gelatin capsules to Coulston guinea pigs for the same exposure period, there was an increased incidence of vertebral malformations (fusion of atlas and axis).

The importance of the type of vehicle used was also demonstrated in rabbit studies. When doses of 200 mg/kg carbaryl were administered by oral intubation, significant increases in omphalocele and decreases in maternal weight gain were noted. Also, with this treatment, a marginal increase in resorptions was observed (Murray et al., 1979). However, the administration of carbaryl by oral capsules in doses ranging between 50 and 200 mg/kg daily had no effect (Robens, 1969).

Other species such as the Golden Syrian hamster (Robens, 1969) and Rhesus monkey (Dougherty et al., 1971) have shown signs of embryoletality. Hamsters administered doses as high as 250 mg/kg carbaryl and Rhesus monkeys administered doses as high as 20 mg/kg carbaryl by gavage had increases in fetal mortality and increases in the rate of spontaneous abortions, respectively.

Although the rat is one of the major species used in the investigation of teratogenicity of most compounds, there is only one study to date concerning the teratogenic effect of carbaryl on rats. Weil et al., (1972) exposed Harlan Wistar rats by dietary intake for different periods of time during gestation (first week, second week, entire gestation) with doses of 20-500 mg/kg. These data revealed no terata as well as no effect on the number of fetuses/dam or the number of dead fetuses. Only doses as high as 500 mg/kg caused a significant decrease in maternal weight gain when administered during the entire gestation period.

Traditionally, investigators have studied the effects on fetuses of exposures to drugs and chemicals during organogenesis, since such exposures can produce malformations. Prenatal

exposure to drugs, however, may also produce functional disturbances. Therefore, in the study conducted in our laboratory, Sprague-Dawley female rats were exposed for 3 months prior to and during the entire gestational period with doses of 1, 10, and 100 mg/kg carbaryl by gastric intubation. Doses of 100 mg/kg produced significant decreases in maternal weight gain, as well as slight decreases in the number of implants and live fetuses per dam. No skeletal, visceral, or external anomalies were noted (Table 3).

**TABLE 3. TERATOGENIC EFFECTS OF CARBARYL
AND/OR
MALATHION IN THE SPRAGUE-DAWLEY RAT**

	Carbaryl (mg/kg)			Malathion (mg/kg)		Carbaryl/ Malathion (mg/kg)	
	1	10	100	1	50	1/1	50/50
Dam weight gain	99.1 ^a	95.8	65.1 ^b	107.2	91.4	61.2 ^b	73.9 ^b
Placental weight	101.4	104.3	78.6	104.3	97.9	68.6 ^b	68.6 ^b
Implants/dam	98.2	100.0	83.9	90.2	99.1	75.9	76.8
Live fetuses/dam	101.8	100.0	83.9	90.2	99.1	55.4	69.6
Resorptions	0.0	0.0	0.0	1.2	0.0	25.0 ^c	12.6 ^c
Hemorrhagic spots	68.4	205.3	173.7	321.1	542.1 ^c	131.6	668.4 ^c

^a Values represent % control

^b Significantly different from control ($p < 0.05$), analysis of variance with Duncan's multiple range test

^c Significantly different from control ($p < 0.05$), Kruskal-Wallis test with chi-square (2x2) contingency tables.

A few studies reported the effects of carbaryl injection into fertile eggs. White Leghorn chick embryos injected with carbaryl into the yolk sacs on various days of incubation produced a variety of abnormalities. When the injection was made on day 4 of incubation with 0.1-3.0 mg/egg, observations made on day 19 showed a dose-response increase in type I abnormalities which include abnormal feathering, micromelia, increased incidence of straight legs, and overall growth retardation (Procter et al., 1976). Additional work by Swartz (1981) revealed that the response (the mortality and subcutaneous edema) after 5 and 12 days of incubation following injection of 1-10 mg/egg on day one was increased. As exposure time increased so did the severity of the toxic response.

The avian embryo is nonplacental and therefore does not have the protection offered by maternal absorption, detoxification, and excretion of foreign compounds (Durham and Williams, 1972).

The results thus far accumulated indicate a difference in the teratogenic potential of carbaryl with regard to the species or the type of vehicle used. Species variation in the metabolism and disposition of carbaryl may account for some differences, since Knaak et al., (1965) reported marked differences in the metabolic fate of carbaryl among mammalian species including man. The dog, which shows the greatest teratogenic effect, at low doses metabolizes carbaryl quite differently from the rat (Knaak and Sullivan., 1967). The rat has major urinary metabolites which include 1-naphthol and hydroxycarbaryl conjugated with glucuronic or sulfuric acids. The major difference in carbaryl metabolism between the rat and the dog appears to be the inability of the dog to metabolize carbaryl to 1-naphthol or hydroxycarbaryl. However, it was found that the dog is able to conjugate 1-naphthol or hydroxycarbaryl when administered as the parent compound (Knaak and Sullivan, 1967).

Various mechanisms for carbaryl's teratogenic effect have been elucidated in chick embryos. Proctor et al. (1976) revealed that the lowering of nicotinamide adenine dinucleotide (NAD) produced a dose-response relationship with increasing teratogenicity by carbaryl in the chick embryo. In the same study, the administration of 0.8 μ moles nicotinamide completely reversed the teratogenic signs and the NAD levels returned to control values. Tryptophan (5 μ moles), a precursor of NAD, was found to be partially effective in alleviating these teratogenic signs. Thus, a link was made connecting a blockade in tryptophan biotransformation and lowered NAD levels. Carbaryl was found to inhibit kynurenine formamidase, which is an enzyme in tryptophan metabolism responsible for converting N-formylkynurenine to kynurenine in the yolk sac membrane and in the mouse liver by 75 and 20 percent, respectively (Moscione et al., 1977; Eto et al., 1980). The inhibition of this enzyme in both instances has a high correlation factor with decreasing NAD levels and producing type I teratogenesis in chick embryos.

REPORTS OF MALATHION TERATOGENICITY

As compared to the number of teratology studies done with carbaryl, there have been only a few studies performed using malathion (Table 4). Khera et al. (1978) administered doses between 50 and 300 mg/kg malathion to the Wistar rat on day 6-15 of the gestational period. They found that only at the 300 mg/kg dose was the maternal body weight significantly different from control. All of the other typical teratological parameters were within control range. However, when Dobbins (1967) examined the effect of malathion on the Wistar rat, various changes were noted. The treatment period ranged from single exposures on day 9 or 10 to multiple exposures on day 8-12 or 12-15. The greatest increase in resorptions and decrease in fetal size was noted following treatment on day 9 with 200 mg/kg malathion; however, no effect was seen in soft tissue abnormalities. When the same dose was administered on day 10, no resorptions were produced, but a 20% increase in hydronephrosis and hydroureter was noted.

TABLE 4. TERATOLOGY STUDIES WITH MALATHION

LABORATORY ANIMAL AND REFERENCE	DOSE (MG/KG) AND ROUTE	TREATMENT PERIOD (DAY)	RESPONSE
Wistar rat (Khera et al., (1978))	300; oral	6-15	dam wt. gain
Wistar rat (Dobbins, 1967)	200; oral	9	resorptions ↓fetal weight
	200; oral	10	hydroureter hydronephrosis
	100; oral	8-12	hydroureter hydronephrosis ↓fetal weight
	100; oral	12-15	hydroureter hydronephrosis
Sherman rat (Kimbrough and Gaines, 1968)	600-900; oral	11	no toxic re- sponse
W. Leghorn chick embryo (Greenberg and LaHam, 1969)	4-6 mg/egg; yolk sac injection	4; 5	type I anomalies
W. Leghorn chick embryo (Walker, 1971)	1-5 mg/egg; yolk replacement	3	type I anomalies
W. Leghorn chick embryo (Proctor et al., 1976)	5 mg/egg; yolk sac injection	4	no toxic response

Multiple treatments with doses of 100 mg/kg on day 8-12 produced decreases in fetal weight; however, no effect was observed in this parameter after exposure to the same dose on day 12-15. Both of these multiple treatments produced slight increases in hydronephrosis and hydroureter. When doses of 600-900 mg/kg malathion were administered orally to the Sherman rat on day 11 of gestation, no effect was produced (Kimbrough and Gaines, 1968). The various effects noted were probably due to the rat fetus being insensitive to malathion on certain days.

Our laboratory conducted a malathion teratology study by oral intubation in Sprague-Dawley rats by examining the effect after an exposure of 3 months prior to and throughout gestation. There was evidence of one significantly different external anomaly, namely hemorrhagic spots on the upper back (Table 3).

There appears to be a discrepancy in the teratogenic potential of malathion in chick embryos which is related to the type of vehicle used. Exposure of the White Leghorn embryos to (4-6 mg/egg) malathion by injection into the yolk sac (Greenberg and LaHam, 1969) or by yolk replacement (1-5 mg/egg) using corn oil as the vehicle (Walker, 1971) produced typical type I anomalies. On the contrary, Proctor et al, (1976) found that no terata were produced after exposure to the same concentrations of malathion using a methoxytriglycol vehicle. A study performed by Greenberg and LaHam (1970) supports their original findings by observing that chick embryos exposed to malathion have significantly decreased levels of tryptophan as compared to control. Additional tryptophan completely reversed the anomalies; however, precursors of NAD only partially reversed this effect.

REPORTS OF INCREASED TERATOGENICITY EFFECTS FROM CARBARYL AND MALATHION IN COMBINATION

Little research has been conducted on the teratogenic effects of multi-exposure to chemical agents in the mammalian species. Experimental evidence indicates that an embryo is more susceptible to an interaction between 2 or more compounds than is the mother (Durham and Williams, 1972). However, there are a few studies involving the interaction of carbaryl and malathion as summarized in Table 5.

Ghadiri and Greenwood (1966) injected chick embryos with carbaryl and malathion singly and in combination at doses of 1-4 mg/egg. After the exposure to the combination, type I teratogenesis was prominent.

Following these studies, Ghadiri et al. (1967) administered carbaryl and malathion singly and in combination in the diets of fertile White Leghorn hens and males for 3 weeks with doses ranging from 75-600 ppm. The liver and kidney tissues of the hens stored a large portion of these pesticides. As the dose of carbaryl and malathion increased, the amount stored increased, and a decrease in hatchability and an increase in type I teratogenesis was observed.

**TABLE 5. TERATOLOGY STUDIES
WITH THE COMBINATION OF CARBARYL AND MALATHION**

<u>LABORATORY ANIMAL AND REFERENCE</u>	<u>DOSE (MG/KG) AND ROUTE</u>	<u>TREATMENT PERIOD (DAY)</u>	<u>RESPONSE</u>
W. Leghorn chick embryo (Ghadiri and Greenwood, 1966)	1-4 mg/egg; yolk sac injection	4	type I anomalies
W. Leghorn hens and males (Ghadiri et al., 1967)	0-600 ppm; diet	0-21	type I anomalies ↓hatchability
Medaka eggs (Solomon & Weis, 1979)	2.5/5.0 ppm carbaryl/ malathion; dipped	0-7	circulatory abnormali- ties ^a

^a This dose produced a synergistic response.

Since insecticides are known to reach the aquatic ecosystem, Solomon and Weis (1979) examined this combination's effect on Medaka eggs (Japanese Kill Fish). Carbaryl (4.0 ppm), as well as malathion (10.0 ppm), were effective in producing circulatory abnormalities such as changes in heart morphogenesis, clotting, and edema in Medaka eggs when administered singly. Carbaryl was 4 times as potent as malathion. In the same experiment, low concentrations of the combination (2.5/5.0 ppm carbaryl/malathion) produced a synergistic response.

A combination study of carbaryl and malathion was performed in our laboratory on Sprague-Dawley female rats. Doses of 1/1 and 50/50 mg/kg carbaryl/malathion were administered by oral intubation for 3 months prior to and during gestation. Both combination doses produced a significant decrease in maternal weight gain during the total gestational period. This effect was predominant during the third week of pregnancy. The change in maternal weight gain and the significant decrease in placental weight are synergistic in comparison to each of the pesticides alone. The decreases in these parameters are related to the fewer number of fetuses/dam due to the resorptions and the implantation failures (Table 3). The lack of a dose-response relationship with the combination is probably related to the large number of resorptions (80%) that one dam had in the low dose combination group. The only anomaly present was external hemorrhagic spots on the upper back with the high dose combination; however, the incidence was the same as with the high malathion dose (Table 3).

SUMMARY AND CONCLUSION

We have demonstrated that the exposure of animals to the combination of carbaryl and malathion, as compared with carbaryl or malathion alone, produced a marked increase in the severity of the teratogenicity. From the data presented in this report, it is worth noting that many contradictions exist in the teratology studies of carbaryl and/or malathion. These discrepancies can be explained through differences in the route of administration, type of vehicle used, length of exposure period, species differences, and metabolic pathways.

Most studies performed in this area do not take into account exposure of more than one compound at a time. In practice, the average consumer may ingest at the same meal several different food products each containing a different pesticide. The synergistic response observed after exposure to the combination of carbaryl and malathion was exemplified by the circulatory anomalies in the Medaka eggs, the type I abnormalities in the White Leghorn chick embryos, and the increases in maternal toxicity and embryoletality seen in the Sprague-Dawley rat.

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TOXICITY STUDIES ON PERCHLOROETHYLENE - ETHANOL INTERACTION

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These studies were undertaken to investigate the interaction of perchloroethylene (PERC) with ethanol (ETH). Groups of randomized male and female Sprague-Dawley rats (40 rats/level/sex) were exposed to various concentrations of PERC - ranging from 100 to 14000 ppm - for a single period of 4 hours. Twenty rats of each level received, 30 minutes after exposure, a single oral dose of ETH (8 mL/kg) diluted with H₂O (50/50, v/v) whereas the remaining rats were kept as PERC control. Both blank control and ETH control rats (20 rats/level/sex) were also exposed to clean air for 4 hours in similar inhalation chambers. All control rats and those surviving the above treatment were sacrificed 15 days after exposure.

The following LC₅₀ data were obtained in rats exposed to PERC alone: 11,484 ppm (C.L.: 10,006 - 13,109 ppm) in males and 10,155 ppm (C.L.: 8,916 - 11,500 ppm) in females. For rats treated with both PERC and ETH, a 50% reduction of LC₅₀ was observed: 5,367 ppm (C.L.: 4,585 - 6,251 ppm) in males and 4,989 ppm (C.L.: 4,310 - 5,770 ppm) in females. Statistical analysis of the above data (two sample z-test) indicated that ethanol produced a significant reduction of LC₅₀ in both sexes ($z = 6.47$, $p < 0.0001$ for males; $z = 6.51$, $p < 0.0001$ for females).

Gross and histopathologic examination of several tissues (adrenals, brain, heart, kidney, liver, lungs, etc.) revealed liver lesions which were assessed according to a 5-point scoring system developed in our laboratory. No lesions were seen in rats exposed to the lowest level whereas liver necrosis with gross vacuolization (ballooning) of liver cells was observed in male rats surviving the highest PERC level. The results obtained indicate that a direct correlation exists between the levels of exposure to PERC and the severity of hepatic damage induced. Furthermore, the liver changes observed in rats exposed to PERC levels between 500 and 6,000 ppm and subsequently treated with ethanol were more pronounced than those induced in animals exposed to PERC alone. No significant lesions were observed in the other tissues examined.

These results indicate that the inhalation toxicity of perchloroethylene is enhanced by orally administered ethanol.

TOXICOLOGY OF NATURAL AND MAN-MADE TOXICANTS IN DRINKING WATER

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INTRODUCTION

A chemical is thought to modify the toxic effects of a second chemical by one of four general mechanisms. Most often considered are pharmacologic interactions, or those situations in which there is a commonality in the receptor through which the toxic chemicals exert their effects. The net result of the interaction depends upon the relative affinity of the chemicals (or their active products) and whether the chemicals act as agonists or antagonists. The second type of interaction is that in which a chemical modifies the uptake, distribution, metabolism, or excretion of a toxic chemical. Third are interactions that depend upon the activation or inhibition of physiologic systems that compensate for or amplify the effects of a second chemical. Finally, there are the cases where one chemical actually reacts with a second chemical within the body to produce a more or less toxic group of chemicals.

The present paper deals with an example of the last type of interaction, that of chemical reaction. Disinfection of drinking water utilizes reactive chemicals, usually oxidizing agents, that react with biochemical components of bacteria, viruses, and parasites to kill these infectious agents. The effectiveness of this approach has been demonstrated by the marked decrease in water-borne infectious disease in this country since the early 20th century. However, it is becoming increasingly apparent that disinfectants also react with organic materials present in water or in the gastrointestinal tract to produce potentially toxic by-products. This was first realized when it was found that chlorination of drinking water gave rise to trihalomethanes (Bellar and Lichtenberg, 1974; Rook, 1974). This area has become increasingly complex in the last 2-3 years as diversity of substrates that are available for reaction has become recognized. As might be suspected, the diversity of toxicologic properties associated with these reactions is also growing. For the purposes of this paper, however, only reactions of chlorine to produce chemicals that possess carcinogenic and mutagenic properties will be considered.

REACTION OF CHLORINE WITH ORGANIC CHEMICALS IN DRINKING WATER

Meier et al. (1983) demonstrated that treatment of humic acid with chlorine produced direct acting mutagens in the standard plate assay of Ames (1975) utilizing Salmonella tester strains. In Table 1 the effect of varying pH on mutagen formation is compared. Mutagen formation and chlorine substitution, indicated by formation of total organic halogen (TOX), is very much favored when the pH is allowed to drift to acid pH following addition of chlorine. Although there is still considerable TOX formed at alkaline pH, the mutagenic activity is sharply reduced. This circumstance is at least partially attributable to the fact that mutagens formed at acid pH are very alkaline labile (Meier et al., 1983). It is notable that trihalomethane formation is favored at alkaline pH and they would not be detected under the test conditions used because of their volatility.

TABLE 1. COMPARISON OF THE pH DEPENDENCE OF MUTAGEN AND CLASTOGEN FORMATION UPON TREATMENT OF HUMIC ACID WITH CHLORINE (HOCl/OCl)

No.	Sample	Tox	Mutagen Activity ^a	
			TA98	TA100
A	Chlorinated Humic (pH: 7.0 -2.8)	414	339 ± 29 (100)	1696 ± 148 (100)
B	Chlorinated Humic (pH: 7.0 - 6.5)	263	62 ± 10 (18)	367 ± 34 (22)
C	Chlorinated Humic (pH: 11.5 - 6.5)	280	N.S. ^b (15)	490 ± 33 (29)
D	Non-chlorinated	0.3	N.S. ^b (15)	N.S. ^b (15)

^a Net revertants/ml of sample, calculated from the linear portion of dose response curve. Numbers in parentheses indicate the percent of activity in sample A.

^b N.S. = Not significant (i.e., less than 2-fold above background) for this experiment. Negative control values were 21 revertants/plate for TA98 and 98 revertants/plate for TA100; the highest dose level was 0.4 ml. Therefore, a N.S. response would be less than 52 revertants/ml for TA98 and less than 245 for TA100.

Mutagenic activity formed is proportional to the degree of chlorine substitution as determined with total organic halogen analyses at chlorine to carbon molar ratios between 0 to 1.0. It is difficult to determine the extent to which this is only a fortuitous relationship since the relative importance of oxidation reactions versus chlorine substitution reactions of

HOCl/OCl⁻ in the production of mutagenic activity has not been established. Interpretation is very clouded by the fact that mutagens formed are very alkaline labile.

More than 40 compounds have been tentatively identified in these reaction mixtures (Coleman et al., 1984). They fall into several classes, the trihalomethanes, chlorinated alkanes, alkenes, aldehydes, ketones, acids, nitriles, and aromatics (Table 2). Among these chemicals are a number of established carcinogens and mutagens and others which have been demonstrated to possess such properties in our own laboratory.

TABLE 2. TENTATIVE IDENTIFICATION OF MAJOR CONSTITUENTS OF CHLORINATED HUMIC ACID^a

<u>TRIHALOMETHANES</u>	<u>KETONES (Cont.)</u>
Chloroform ^c	1,3-Dichloro-2-Butanone
	1,1-Dichloro-2-Butanone
	3,3-Dichloro-2-Butanone
	1-Chloro-3-Buten-2-One
	3-Chloro-3-Buten-2-One
	Dichloro-3-Buten-2-One
	Trichloro-3-Buten-2-One
	Tetrachloro-3-Buten-2-One
	Pentachloro-3-Buten-2-One
	Trichlorocyclopentenedione
<u>ACIDS</u>	
Dichloroacetic Acid	
Trichloroacetic Acid	
<u>ALDEHYDES</u>	<u>NITRILES</u>
Dichloroacetaldehyde	Chloroacetonitrile ^c
Trichloroacetaldehyde	Dichloroacetonitrile ^b
Dichloropropanal	Trichloroacetonitrile ^c
Trichloropropanal	Dichloropropenenitrile
2-Chloropropenal	Dichloropropenenitrile
2,3-Dichloropropenal	Trichloropropenenitrile
3,3-Dichloropropenal ^b	
2,3,3-Trichloropropenal	
Trichlorobutanal	
Dichlorobutanal	
<u>KETONES</u>	<u>AROMATICS</u>
1-Chloro-2-Propanone	2,4,6-Trichlorophenol
1,1-Dichloro-2-Propanone ^b	Trichlorodihydroxy Benzene
1,3-Dichloro-2-Propanone ^b	Tetrachlorothiophene
1,1,1-Trichloro-2-Propanone ^b	
1,1,3-Trichloro-2-Propanone	<u>ALKANES AND ALKENES</u>
1,1,1,3-Tetrachloro-2-Propanone	Hexachloroethane ^c
1,1,3,3-Tetrachloro-2-Propanone	Pentachloropropene ^b
Pentachloropropanone ^b	Tetrachlorocyclopropene
3-Chloro-2-Butanone	Hexachlorocyclopentadiene
1,1,1-Trichloro-2-Butanone	
1,1,3-Trichloro-2-Butanone	

^a Data obtained from Coleman et al. (1984).

^b Chemicals identified as mutagens

^c Chemicals identified as carcinogens

Despite the fact that a number of mutagenic chemicals have been identified, to this point only about 1.5% of the total mutagenic activity can be accounted for by chemicals for which both quantitative analyses and mutagenic characterization have been possible. Admittedly, this is somewhat of an artifact due to the lack of appropriate standards and the time lag associated with getting these chemicals synthesized and tested. Nevertheless, this figure does provide some estimation of the complexity of the overall problem. Adding to this complexity is that the formation of mutagenic activity and the identity of products can be markedly changed by incorporating trace quantities of Br^- into the reaction mixture. As Br^- is increased to a molar ratio of 0.1 to the chlorine added, there is an approximate doubling of the mutagenic activity (Meier, unpublished observations). As would be predicted from the well-known ability of HOCl to activate Br^- to HOBr , bromination reactions begin to predominate. As can be seen in Table 3, brominated compounds become very evident at ratios of Br^- to HOCl of 0.01 and progressively increase until completely brominated compounds predominate at ratios above 0.1.

TABLE 3. COMPOUNDS IDENTIFIED^a IN METHYLENE CHLORIDE EXTRACTS OF HUMIC ACIDS TREATED WITH CHLORINE^b WITH VARYING CONCENTRATIONS OF BROMIDE (Br^-)^c

Compounds	Molar Ratio of Br^-/C Where Compound Concentration Is Highest ^d
1,1,1-Trichloro-2-Propanone ^e	0
Pentachloro-2-Propanone ^e	0
Chloroform	0.01
Trichloroacetonitrile	0.01
Bromodichloromethane	0.01
Dichloroacetonitrile	0.01
1,1-Dichloro-2-Propanone	0.01
Bromochloromethane	0.05
Bromochloroacetonitrile	0.1
Dibromochloromethane	0.1
Bromochloroacetonitrile	0.1
1,1,1-Bromodichloro-2-Propanone ^e	0.1
1,1,1-Dibromochloro-2-Propanone ^e	0.1
Bromoform	0.5,1
Tetrabromomethane	1
1,1,1-Tribromo-2-Propanone ^e	1

^a 60M x 0.25 MM I.D. DB1 fused Silica column

^b $\text{Cl}:\text{C}$ Molar ratio 1:1

^c Data obtained from Coleman et al. (1984)

^d Applicable only to ratios tested, concentration is RIC area

^e Standard not available, identified by visual interpretation of mass spectrum

BY-PRODUCTS OF CHLORINE PRODUCED IN VIVO

Drinking water in the U.S. is treated to a residual. This means that water consumed by the public would usually contain between 0.5 and 2 mg/L of disinfectant. It is obvious that the variety of potential substrates for chlorine available within the gastrointestinal tract adds another dimension to this problem. The question naturally arises "can interactions of chlorine reactions with substrates present in the GI tract result in products with carcinogenic and mutagenic properties?"

Experiments sponsored by our laboratory show that OCl^- and ClNH_2 administered orally increase spermhead abnormalities in B6C3/F₁ mice (Meier et al., 1983). An increased percent of abnormal sperm was seen at 3 weeks following dosing, but not at 1 and 5 weeks, consistent with an effect at the spermatocyte stage of spermatogenesis. HOCl was without effect in this system. It is unlikely that OCl^- is capable of reaching the testis because of its reactivity. Therefore, it is felt that these data are evidence for the formation of highly mutagenic chemical(s) in the gastrointestinal tract. It should be noted that conditions of pH responsible for the formation of mutagens in vivo are the opposite of those observed in reactions with humic acid. The fact that the same result was observed in two experiments, with OCl^- (pH 8.5) and with ClNH_2 (pH 9), indicates a degree of consistency in the results.

To document the extent to which chlorination by-products can be formed in the gastrointestinal tract, Mink et al. (1983) intubated rats with relatively high doses of OCl^- (ca. 150 mg/g) and observed the appearance of by-products in the stomach contents and plasma. Table 4 lists the products identified by GC/MS when animals were treated with a chlorine solution containing Br^- . Within the stomach contents, many of the same products seen in the reactions with humic acids were identified but could not be meaningfully quantitated. Primarily, we saw halomethanes, haloacetoneitriles, and halogenated acetic acids.

To what extent can the spermhead abnormalities be accounted for by these products? Not at all, it would appear. Topham (1980) demonstrated that chloroform is negative in this bioassay. Our laboratory has tested the haloacetoneitriles identified in these experiments in the mouse spermhead assay at much higher doses than would be expected to be formed in the G-I tract and found that they were negative as well (Bull et al., unpublished results). Although we have not yet tested this possibility, it seems highly unlikely that these effects could be attributed to the highly polar haloacetic acid products formed both because of a lack of intrinsic activities in other mutagenesis test systems and on pharmacokinetic grounds.

TABLE 4. HALOGENATED PRODUCTS OBSERVED IN THE RAT STOMACH AND BLOOD PLASMA FOLLOWING ACUTE TREATMENT WITH CHLORINE AND KBr^a

<u>Compound</u>	<u>Stomach Content</u>	<u>Plasma</u>
Chloroform	+ ^b	-
Bromoform	+	+
Bromodichloromethane	+	-
Dibromochloromethane	+	+
Dichloroacetonitrile	+	-
Trichloroacetonitrile	-	-
Dibromoacetonitrile	+	-
Dichloroacetic Acid	+	-
Dichloroacetic Acid	+	-
Trichloroacetic Acid	+	-
Dibromoacetic Acid	+	-
Dibromomethane	+	-

^a Treatment involved gavage with total dose of 48 mg Cl (as NaOCl) and 32 mg Br⁻(as KBr). Analyses conducted 1 h after dosing.

^b Positive sign (+) indicates identification of the indicated compound by GC/MS. Negative sign (-) indicates compounds were below detection limit.

However, it is clear that the haloacetonitriles do possess significant biological properties (Table 5). Two haloacetonitriles, dichloroacetonitrile and bromo-chloroacetonitrile, are mutagens in *Salmonella*. In addition, the dibromo-, the bromochloro-, the trichloro-, and the monochloro- acetonitriles act as tumor initiators in the mouse skin (Bull et al., unpubl. results). The ability of these same four compounds to produce lung adenomas has also been demonstrated in Strain A mice with oral administration of 10 mg/kg 3 times weekly for 8 weeks.

CONCLUSIONS

The major conclusion of this work is that toxic properties associated with reactive chemicals such as the drinking water disinfectants must be viewed as being potentially quite diverse. It is clear that a diversity of mutagenic and carcinogenic chemicals in addition to the trihalomethanes are formed both in drinking water and in vivo through reactions of chlorine with endogenous substrates. Evaluation of the overall toxic effects of chlorine must deal with the diversity of products that can be formed and the fact that these products have their own intrinsic toxicologic properties. To complete the situation, it is also quite clear that the nature of the products formed will vary with the substrates available in the diet. Amino acids have been shown as substrates that can give rise to haloacetonitriles

TABLE 5. ACTIVITY OF HALOACETONITRILES IN MUTAGENESIS AND CARCINOGENESIS SCREENING PROCEDURES

	<u>Salmonella^a</u>	<u>SCE Induction^a</u>	<u>Tumor Initiation Mouse Skin^b</u>	<u>Mouse Lung Adenomas^c</u>
Chloroacetonitrile	- ^d	+	+	++
Dichloroacetonitrile	++	±	±	±
Trichloroacetonitrile	--	++	+	+
Dibromoacetonitrile	--	+++	+++	+
Bromochloroacetonitrile	+++	+++	++	+++

^a Meier et al., Personal Communication

^b Bull et al. (1982^b) Toxicologist 2:326

^c Bull et al. (1983) Unreported Data

^d Indication of negative (-) or relative degree of positive response (+)

(Biebar and Trehly, 1982). They may also give rise to a group of organic chloramines (Scully et al., 1983), an example of which is N-chloropiperidine, extremely potent in producing spermhead abnormalities in mice.

In summary, reaction of chlorine in biological systems will necessarily give rise to a very complex mixture of organic chemicals. Whether the toxic chemicals that arise from reactions of chlorine are produced in sufficient quantities to present a meaningful hazard to human health remains to be determined. However, by-products of chlorination are the most common and plentiful organic chemicals identified in drinking water. Their levels outstrip the levels of industrial chemicals by 1-2 orders of magnitude in finished waters taken from surface sources. Finally, we must recognize that other potential disinfectants are also reactive chemicals (chloramine, chlorine dioxide, and ozone). Therefore, analogous problems will also arise from their use. The only resolution of this problem is a systematic study of the reactions of these chemicals, identification of the major by-products, and characterization of their toxicologic properties. Once these are established, the toxicity of these extremely complex mixtures can be meaningfully and usefully studied.

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ASPECTS OF SOLVENT TOXICITY IN MIXTURES

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INTRODUCTION

Over the last few decades, great advances have been made in establishing approximations of the safe and permissible limits of incidental or occupational exposure for the protection of workers engaged in manufacturing and processing of industrial volatile solvents. Information gathered from experimental studies with laboratory animals along with cumulative experiences of many industrial studies provides the data base for these estimations of risk and potential health hazard associated with each of the most commonly used solvents (and other chemicals). In general, concern for occupational exposure restricted studies of the safety evaluations to those time intervals and concentrations of substances consistent with usual working conditions. As a result, most of the literature dealing with volatile agents is derived from studies of single agent exposure (or administration) aimed at providing safety data and hazard evaluation for the work environment. Consequently, these often described results of either acute high dose mortality data (LD₅₀, LC₅₀) or long term (chronic) low concentration exposures yielding subtle, if any, alteration from normal. In either case, the data obtained provide guidelines for establishing hazard indices. Animal studies coupled with any known human exposure at various safe and sometimes lethal levels of chemical agents are compiled, evaluated, and used as a basis for recommended short-term exposure limits (STEL), i.e., the maximal concentrations acceptable for 15 minutes, no more than four times a day, without producing irritation, tissue damage, or hazardous necrosis; or the threshold limit value (TLV-TWA) time-weighted average, i.e., an average exposure level a worker can be exposed to for an 8 hour work day

*Presenter

over an indefinite period of time believed to be without any hazard to health. These kinds of studies continue to serve as indices for potential hazards associated with human exposures.

In this presentation we will deal with chemicals produced in the United States in excess of 1 billion pounds per year. It is presumed that chemicals produced in such high volume would be those most likely to present health hazards under certain conditions of exposure. A recent EPA document, "Perspectives on the Top Fifty Production Volume of Chemicals" (July 1980), lists the Top 50 Chemicals (See Table 1).

TABLE 1. CHEMICAL PRODUCTION TOP 50

1	sulfuric acid	26	ethylene oxide
2	lime	27	carbon dioxide
3	oxygen	28	ethylene glycol
4	ammonia	29	ammonium sulphate
5	nitrogen	30	butadiene
6	ethylene	31	p-xylene
7	chlorine	32	carbon black
8	sodium hydroxide	33	cumene
9	phosphoric acid	34	acetic acid
10	nitric acid	35	phenol
11	sodium carbonate	36	sodium sulfate
12	ammonium nitrate	37	calcium chloride
13	propylene	38	aluminum sulfate
14	benzene	39	cyclohexane
15	urea	40	acetone
16	ethylene dichloride	41	propylene oxide
17	toluene	42	acrylonitrile
18	ethyl benzene	43	isopropyl alcohol
19	vinyl chloride	44	adipic acid
20	styrene	45	vinyl acetate
21	formaldehyde	46	sodium silicate
22	methanol	47	acetic anhydride
23	xylenes	48	sodium tripolyphos
24	terephthalic acid	49	titanium dioxide
25	hydrochloric acid	50	ethanol

Chemicals are in order of production volume, approximately 100×10^6 for sulfuric acid to 1×10^6 lbs. for ethanol

Although eleven of the top twelve are inorganic (elements, mineral acids and bases or salts), 31 of the 50 are organic, mostly monomers of plastics, rubbers or fibers; also fertilizers,

antifreeze and intermediates. Xylene appears twice - once as mixed xylenes which is mostly meta and also as p-xylene, although toxic levels are not distinguished.

All of the organic solvents on our list evaporate, producing vapor which unfortunately cannot be seen and often cannot be smelled at the TLV, e.g. ethylbenzene, ethylene dichloride, and propylene oxide. How much propylene oxide is 20 ppm or 50 mg/m³? We have calculated the amounts for the average sized laboratory (10 x 24 x 36 ft (8640 ft³) for each of the solvents, see Table 2, column 8. For propylene oxide this is 12 grams. The list ranges from 1 gram of acrylonitrile to about 500 grams of ethanol.

TABLE 2. ORGANIC CHEMICALS IN TOP 50 RANKED BY BOILING POINTS

Organic Substance	TWA ppm	TWA mg/m ³	STEL mg/m ³	Flash C	M. P. C	B. P. C	Mol. Wt. g/mole	TWA* grams	Vap. Pres. mm Hg.
1 ethylene					-169	-104	28		52400
2 carbon dioxide	5000	9000	27000		s	-79	44	2202	48250
3 propylene					-182	-47	42		8666
4 formaldehyde	2	3			-92	-20	30	1	4177
5 vinyl chloride	5	10			-160	-14	63	2	3214
6 butadiene	1000	2200	2750		-109	-5	54	538	2260
7 ethylene oxide	10	20			-111	11	44	5	1290
8 propylene oxide	20	50	360	-37	-112	34	58	12	532
9 acetone	750	1780	2375	-20	-94	57	58	435	221
10 methanol	200	260	310	12	-98	65	32	64	122
11 vinyl acetate	10	30	60	-6	-93	72	86	7	108
12 acrylonitrile	2	4.5		0	-84	77	53	1	110
13 ethanol	1000	1900		9	-114	79	46	465	57
14 benzene	10	30	75	10	6	80	78	7	95
15 cyclohexane	300	1050		-18	6	81	84	257	98
16 isopropyl alcohol	400	980	1225	12	-89	83	60	240	43
17 ethylene dichloride	10	40	60	15	-35	83	99	10	82
18 toluene	100	375	560	6	-95	111	92	92	28
19 acetic acid	10	25	37	40	17	118	60	6	3
20 ethyl benzene	100	435	545	20	-95	136	106	106	10
21 p-xylene	100	435	655	30	13	137	106	106	9
22 acetic anhydride	5	20	16	54	-73	139	102	5	5
23 xylenes	100	435	655	29	-47	139	106	106	8
24 styrene	50	215	425	32	-31	145	104	53	7
25 cumene	50	245	365	46	-96	152	120	60	4
26 ethylene glycol	125	125	325	115	-13	198	62	31	0
27 phenol	5	19	38	79	43	182	94		
28 urea					135	d	60		
29 terephthalic acid					s	300	166		
30 adipic acid				196	152	338	146		
31 carbon black		3.5	7		s	4200	12	1	

s = sublimes

d = decomposes

* in 8640 cubic feet

The following equations are used for calculation of TWA in laboratories (8640 ft³ is 10,000 moles at 25°C):

1 mole at 25°C is 24.4654 liters
 1 cu. ft. is 28.32 liters
 8640 cu. ft. is 244,684.8 liters
 8640 ft.³ x 28.32 l/ft.³ is 244,684.8 liters is 10,001 moles

$$\begin{array}{ccccccc} \text{ppm} & & \text{Mol. Wt.} & & & & \\ \frac{20 \text{ parts}}{10^6 \text{ parts}} & \times & \frac{58 \text{ g}^*}{\text{mole}} & \times & \frac{10^4 \text{ moles}}{\text{room}} & = & 11.6 \text{ g} \end{array}$$

* grams propylene oxide; TWA 20 ppm

These compounds are liquids at room temperature, and inhaled toxicity is dependent upon their physical properties. For example, a compound which has a relatively high TLV can still be hazardous if it readily vaporizes at room temperature. These equations include vapor pressure, a measurement of the partial pressure the solvent exerts at 25°C. Vapor pressures which have higher numerical values are generally more easily volatilized than those with lower values, e.g., acetone is about twice as volatile as methyl ethyl ketone. The more volatile substances will be more concentrated in inhaled vapors.

Two factors are important in considering volatility. The vapor pressure tells us what maximum concentration can be reached, evaporation rates tell us how fast the maximum can be reached. Vapor pressure can be expressed as % or as ppm. In Table 3 we compare TWA in ppm with the concentration of saturated vapor in ppm. Note that the relation of saturated vapor to TWA is a measure of the potential inhalation hazard. We call this ratio the inhalation hazard potential (IHP). Note that it varies from 72 thousand for acrylonitrile to one for ethylene glycol. Also, as expected, vapor pressures correlate well with boiling points; only ethyl and isopropyl alcohols, acetic acid, and its anhydride are lower than expected.

Although p-xylene is "7 1/2 times as toxic" as acetone (compared TWA), acetone has 27 times the vapor pressure of xylene and is potentially a greater hazard. Acetone also evaporates 4.5 times as fast as p-xylene. Evaporation rates vary greatly with test conditions; they depend upon the temperature and velocity of

ventilating air as well as the vapor pressure and surface tension of the solvent. In general, evaporation rates correlate well with vapor pressure. Only methanol and acetic anhydride evaporate slower than expected from their vapor pressures, while toluene and acetic acid evaporate faster.

TABLE 3. ORGANIC SOLVENT INHALATION AND FLAMMABILITY HAZARD POTENTIAL

Substance	TWA	Vp	LEL	UEL	Vp/TWA	LEL/TWA	Vp/LEL
Propylene Oxide	20	700250	20000	220000	35013	1000	35
Acetone	750	290237	25500	128000	387	34	11
Methanol	200	160531	67200	365000	803	336	2
Vinyl Acetate	10	142632	26000	134000	14263	2600	5
Acrylonitrile	2	144763	30500	170000	72382	15250	5
Ethanol	1000	75237	32800	189500	75	33	2
Benzene	10	124999	4000	71000	12500	400	31
Cyclohexane	300	128605	12600	77500	429	42	10
Isopropyl Alcohol	400	56645	20200	118000	142	51	3
Ethylene Dichloride	10	108012	62000	159000	10801	6200	2
Toluene	100	37164	12700	67500	372	127	3
Acetic Acid	10	4157	54000		416	5400	0
Ethyl Benzene	100	12500	10000	67000	125	100	1
P-xylene	100	11519	11000	70000	115	110	1
Acetic Anhydride	5	6658	29000	103000	1332	5800	0
Xylenes	100	10851	10000	60000	109	100	1
Styrene	50	9599	11000	61000	192	220	1
Cumene	50	5906	9000	65000	118	180	1
Ethylene Glycol	125	175			1		

TWA = Threshold Limit Value - Time Weighted Average in ppm

Vp = Concentration of Saturated Vapor in ppm (Vapor pressure)

LEL = Lower Explosive Limit in ppm

UEL = Upper Explosive Limit in ppm

While considering toxicity, we should not overlook the hazard of fire or explosion. Table 3 also lists the lower and upper explosive limits in ppm. All the TWA's are well below the Lower Explosive Limits. In fact, the LEL is 33 times, usually 100 to 1000 times, and ranges up to 15,000 times the TWA.

In case we have given the impression that toxic effects can be predicted from chemical activity, vapor pressure, and rate of evaporation, we hasten to point out that carbon black, amorphous carbon, is chemically inert at 25°C, has an extremely low vapor pressure (estimated to be less than 10^{-50} mm Hg) and does not evaporate but sublimates at 4800°C. And yet carbon can be present in high concentrations as a dust, and worse yet, other substances can be absorbed on the surface of the dust particles and carried into the lungs. Coal dust is 80% (anthracite) to 25% (lignite) fixed carbon.

It is important to recognize that chemicals, even when they start as relatively pure materials, being chemicals or solvents, are readily contaminated with other substances. In use, chemicals or solvents consist of mixtures of varying composition and the effects noted in most studies are of single agent exposure (as previously stated) and may at best suggest likely target organ specificity or minimal toxicity (Couri and Abdel-Rahman, 1977).

In the recent literature there have been many reports correlating chemical exposure to alterations in organs, organelles, enzyme levels, metabolites, body fluid components, and other vital functions of the body. Research has attempted to determine the levels of chemical exposure at which these alterations will occur. Investigators have also attempted to determine at what levels of exposure the damage produced by these agents is reversible and at what levels irreversible damage occurs and the time frames involved.

In an evaluation of the toxicity of a solvent or a single component of a mixture, important considerations include the following: A) whether the parent compound or metabolite(s) is responsible for the toxicity, B) if it is the metabolite(s), then the rate of formation and distribution of the active toxic metabolite must be considered, and C) the factors affecting the metabolism and excretion of the toxicant or toxic metabolite. In addition, other external factors increasing or decreasing any of these must also be taken into consideration. The following section on single or mixed chemical exposure provides a sampling of examples of these factors.

SINGLE AND MIXED CHEMICAL EXPOSURES

Many investigators have demonstrated that exposure to a chemical such as a single solvent may lead to a variety of alterations in vital bodily functions as mentioned previously. In 1978, Engstrom et al. reported that exposure to xylene via inhalation (e.g. as in paint thinners) showed a high correlation between urinary methylhippuric acid (mg/g of creatinine) and the time weighted average. It is well known that xylene is biotransformed by the body to toluic acid, which is then conjugated with glycine and excreted as urinary methylhippuric acid. Engstrom showed that 3 hours after a single exposure to xylene, a high concentration of methylhippuric acid was present in the urine and elimination of methylhippuric acid was continuous for the next 20 hours following the exposure to xylene. He was able to demonstrate low levels of excretion of methylhippuric acid for at

least 72 hours following exposure, indicating that xylene was retained and distributed in the body and being released slowly and metabolized for days following the initial exposure, suggesting a secondary excretion phase of methylhippuric acid and supporting the idea that xylene was indeed retained by the body after the initial exposure. As mentioned, Engstrom was able to correlate the methylhippuric acid concentration with the amount of creatinine present in the urine. For example, at 25 and 50 ppm of xylene exposure, levels of approximately 350 and 665 mg methylhippuric acid/g creatinine, respectively, were present in urine. From these data, it was concluded that methylhippuric acid excretion was a linear function of exposure and could be used as an indicator of xylene exposure. Engstrom was not able to correlate blood levels of xylene with expired air because xylene is highly soluble in blood which favored xylene retention in blood circulation, leaving insignificant amounts to be expired into the atmosphere. Exhaled air samples can be used only for a rough estimation of momentary exposure in the work environment. Since xylene is highly soluble in blood, this would account for the persistent levels of xylene in the body mentioned earlier. Engstrom was able to use the blood levels of xylene as an indicator of the body burden levels for different concentrations of xylene. Neither exhaled air samples nor venous blood taken during or after a work day at given intervals after termination of exposure produced particularly accurate information on the average amount of xylene in ambient air. The best indicator for xylene exposure was the level of methylhippuric acid expressed as mg per gram of creatinine excreted.

More recently, there have been reports by DiVincenzo et al. (1982), O'Donoghue et al. (1982), and Shifman et al. (1981) on the neurotoxicology of 5-nonanone. In these studies, the conclusion was drawn that 5-nonanone was not responsible for the neurotoxicity demonstrated in rats. The neurotoxins were postulated to be the γ -diketones such as 2,5-hexanedione and 2,5-nonanedione as a consequence of metabolism of 5-nonanone. The 2,5-hexanedione has been shown to be a potent neurotoxicant metabolite of hexane, and, conversely, the 2,5-nonanedione has now been shown to be the neurotoxicant of 5-nonanone. The available evidence suggests that the γ -diketone structural feature is critical in producing neurological dysfunction in laboratory animals. In the study of DiVincenzo et al. (1982), rats were given ^{14}C -5-labelled 5-nonanone and the metabolites in urine and plasma were identified by gas chromatography/mass spectrometry. In the plasma, 5-nonanol, 2-hydroxyl-5-nonanone, and 2,5-nonanedione were identified; in addition 5-nonane was present. While metabolites identified in the urine were similar to those described previously from n-hexane and methyl-n-butyl

ketone, i.e. methyl n-butyl ketone (2-hexanone), 2,5-hexanedione, 5-hydroxy-2-hexanone, 2,5-hexanedione, 2-hexanol, and γ -valerolactone, 5-nonanone was not present in the urine (Couri and Milks, 1982). The percentage of radioactivity recovered in their studies was 98% of the dosage administered. Their findings suggested that the metabolites of 5-nonanone were dose dependent and occurred by metabolic pathways similar to those elucidated previously for the hexacarbonyls, n-hexane, and methyl n-butyl ketone. In the O'Donoghue study, methyl heptyl ketone (MHK) was one of a series of commercial grade, aliphatic solvents examined (i.e. diisooamyl ketone, diisobutyl ketone, methyl isoamyl ketone, di-n-propyl ketone, and n-heptane) that produced toxic neuropathy in laboratory rats. The neuropathy produced by MHK was clinically and morphologically similar to that reported for n-hexane, methyl butyl ketone (MBK), and 2,5-hexanedione. Analysis of MHK revealed that it was a mixture of approximately 72% 5-methyl-2-octanone (5m2O), 12% 5-nonanone, 0.8% MnBK, and 15.2% C₇-C₁₀ ketones and alkanes. Experimentation with purified samples of 5m2O and 5-nonanone resulted in clinical neurotoxicity with 5-nonanone at doses 4.3 times higher than in commercial mixture. Neurotoxicity was not produced with 5m2O. By combining 5m2O and 5-nonanone in the same proportion as in MHK, the degree of neurotoxicity seen with MHK was reproduced suggesting that 5m2O potentiated 5-nonanone approximately six fold. When an equimolar dose of methyl ethyl ketone was substituted for 5m2O, little or no potentiation was observed. However, Shifman et al. (1981) reported that rats chronically exposed to 5-nonanone showed clinical neuropathy characterized by giant axonal swelling filled with neurofilaments, similar to those produced by hexane, methyl butyl ketone, 2,5-hexanedione and other γ -diketones. In addition, they found that the toxicity of 5-nonanone was enhanced by simultaneous exposure to the microsomal enzyme inducing agent methyl ethyl ketone.

In recent years, investigators have reported polyneuropathies occurring in acute and chronic inhalers of glues and paint thinners. Dyro (1978) investigated solvents acting independently of other solvents and then acting together. His objective was to determine whether or not solvents acting together would have an additive or greater effect with respect to damage than either solvent acting alone. He established the fact that many agents such as n-hexane, acrylamide, and methyl butyl ketone have been shown to cause nerve damage thus affecting nerve conduction velocities and prolonged distal motor latencies (Allen et al., 1975; Herskowitz et al., 1971; Garland et al., 1968). Dyro investigated the mixed solvent effects of methyl ethyl ketone (MEK) with acetone and toluene via the inhalation route. He based his studies on the finding of Altenkirch et al. (1979)

showing that methyl ethyl ketone was capable of enhancing the toxic effects of methyl butyl ketone and n-hexane. Dyro first studied a glue (Supergrip 335 SAC) used in an Ohio manufacturing company that was composed of 51% MEK and 27% toluene. The workers studied were usually exposed to approximately 10 ppm MEK and 25 ppm toluene. For a normal day, the threshold limit values (TLV) for MEK and toluene were then 100 ppm and 200 ppm, respectively. Dyro found that workers had significant neuropathies and proposed that MEK was not the cause of the neuropathy. He suggested that MEK was enhancing the effects of toluene, an agent known to cause neurotoxicity. It was noted that when the workers ceased employment, the symptoms persisted for a year. On follow-up examinations the majority of the symptoms gradually disappeared within two years, with the exception of a few symptoms which could be attributed to other sources. It should be stated that the values for MEK and toluene were estimated and that the solvent vapor concentrations at times reached the TLV.

In another study, Dyro examined a glue containing MEK and acetone. The workers were exposed to 20-180 ppm MEK and 30-250 ppm acetone. The TLV for MEK was then 200 ppm and 1000 ppm for acetone. From the study of these workers, Dyro was able to show that an MEK and acetone combination produced increased neurological damage compared to that expected of either solvent acting independently. Two years after cessation of exposure to these solvents, some workers still showed mild neuropathy, suggesting MEK and acetone was more damaging than either alone, or even MEK and toluene. There have been several other reports of industrial toxicity which involved the presence of MEK in a mixture with other solvent(s). For example, with 10% 2-nitropropane-MEK 500 ppm (Elkins, 1959); MEK and an unsaturated ketone impurity (Smyth, 1956). In each of these cases workers presented symptoms which were of greater severity than could be accounted for by any of the individual components.

We have demonstrated that the exposure of animals to the combined methyl butyl ketone/methyl ethyl ketone vapors, as compared to methyl butyl ketone alone, produced a marked increase in the severity of the polyneuropathy and an elevation and a prolongation of the methyl butyl ketone blood levels in exposed animals. We suggest that the exposure to other volatile solvent mixtures as compared to the individual substances alone may similarly produce an increased toxicity in both occupational and inhalational abuse of industrial solvent exposures. Several studies describing the increased toxicity resulting from the

intentional inhalation of toluene and hexane compared to toluene alone were reviewed earlier (cited in Couri and Abdel-Rahman, 1978).

Similar events have been reported for ketones in combination with butyl, ethyl, amyl acetates and other solvents (Browning, 1965; Couri et al., 1974; Couri et al., 1976; Prockop et al., 1974), while other studies have indicated that workers exposed to 1000 to 2000 ppm acetone for many years show no injury or, at worst, a dull headache with temporary anorexia.

The well-known bone marrow injury related to benzene exposure was in some earlier reports thought to be a toxicity of toluene. It was later proven to be the presence of benzene contamination in toluene which was responsible for the myelotoxic events (Hamilton & Hardy, 1974). This example of marrow toxicity caused by very low levels of benzene in toluene can be looked upon as an enhanced toxicity of low concentrations of benzene when combined with toluene. In the foregoing discussion of solvents we have exclusively looked at specific targets of chemicals in general. We should not neglect the health status of individuals exposed to chemicals or solvents. Host factors impacting significantly in relationship to chemical exposure are the following: A) age, B) overall health status, C) immune system, D) fatigue, E) life style, F) race, G) sex, and H) genetic history. We will briefly discuss each of the factors as related to chemical exposure.

HOST FACTORS

AGE: It is generally felt that as an individual ages there is a gradual decrement in physiologic processes, especially beyond age 40. This would then make for greater susceptibility for toxicity upon exposure to chemicals. Age, in and of itself, is not of any great significance in the evaluation of an individual's response to chemical exposure(s).

Of great importance to workers is an effect such as the potential impairment of reproductive capacities which would result from exposure to certain solvents or chemicals, e.g. dibromochloropropane (DBCP) and ethylene oxide crown ethers.

Relative to age is the state of preservation of healthy organ function. A certain percentage of individuals beyond 40 years of age develop degenerative disease symptoms which are sometimes difficult to differentiate from those symptoms resulting from chemical exposure (Dyro 1978; Browning 1968). For example, complaints such as weakness, fatigue, headaches, malaise

would represent physiological defects attributable to natural processes or exposure to a variety of solvents and/or chemicals (Couri et al., 1976; Couri and Milks, 1980).

HEALTH STATUS: Workers with healthy, well-functioning physiologic systems are better able to handle chemical exposures. The uptake, distribution, metabolism, and excretion of most chemical exposures at low to moderate concentrations are readily processed and excreted with little or no consequences (Carpenter et al., 1949). In contrast to this, individuals exposed to the same chemicals but having impaired respiratory, hepatic and/or renal function would show severe and possibly irreversible adverse effects attributable to the decreased organ function(s) (Kleinnecht et al., 1980).

There are many solvents (also medicinal agents) capable of altering the rate of liver biotransformation activity and subsequent excretion of chemicals (Odkuist et al., 1979). These alterations might then produce an increase or decrease in the relative toxicity of chemicals upon exposure. The factor health status can be approached from many viewpoints when discussed in relationship to exposure to chemicals. First of all, one can view it from the aspect of the ability to metabolize and inactivate compounds that are considered harmful to the body. For instance, the inability to metabolize the solvent xylene would lead to many complications in an individual expressed as severe fatigue, weakness, headache, and G.I. discomfort (Dyro, 1978). Hence, liver function would be a significant factor in relationship to health status. In conjunction with liver function, kidney function is also vital and similar in many respects to liver in relationship to the metabolism and excretion of chemical compounds. Also, an important consideration is the presence or the exacerbation of disease by exposure to chemicals (Kronevi et al., 1979; Couri and Milks, 1980). It is also possible that a disease that might have been silent for many years can be induced to surface and cause significant discomfort and illness. Another possibility is that these compounds may manifest actions via the CNS and may cause any alteration in the nervous system to proceed to a higher level of discomfort, agitation or confusion. Thus, it is important when conducting a study on exposure to chemicals that a complete history and physical be given to an individual prior to the study; otherwise, any preexisting alterations in body function would bias the data and yield incorrect results. If any system in the body is not functioning properly, chemical exposure may or may not cause an increased alteration in the system mentioned.

IMMUNE SYSTEM: For many years investigators thought that toluene was the substance that caused bone marrow suppression. But it was not until Hamilton and Hardy in 1974 proposed from available data that toluene was not the cause of the bone marrow damage but rather the contaminant benzene which was present in toluene. As a result of this finding, this spurred many years of research into benzene and analogs capacity to induce damage to the immunological components. Studies have been able to link solvent exposure with leukemia in rubber workers. McMichael et al. (1975) reported an epidemiological study in which there was an association between leukemia and work places involving exposure to chemical solvents. Other studies (Aksoy et al., 1974; Wolf et al., 1981) have shown that chemical solvents, mainly benzene and its homologues, were able to cause neutropenia and anemia. Therefore, if an individual had a subclinical immunological disturbance, then exposure to chemicals and solvents may exacerbate these disturbances to epic proportions (Moszcynski, 1980). These individuals, therefore, may be more susceptible to microbial and other infections that could lead to life threatening conditions.

FATIGUE: From common sense, it is understood that if a person is fatigued he or she may be more susceptible to chemical toxicity due to the fact that reaction time steadily decreases with time. If we were to compare an individual working eight hours to an individual working 12-16 hours in the same chemical environment, then the individual working 12-16 hours would be more likely to be stressed and fatigued than the individual working 8 hours (Capurro, 1979). The individual working 12-16 hours would be exposed to the chemical for a longer time and may not be aware of the chemical effects because of fatigue (Penderson et al., 1980). This is not to say that the individual who only worked eight hours would not be fatigued, but his relative fatigue would be significantly less than the individual working 12-16 hours. In addition, the individual working more hours would be most likely to have more damage on the average than the individual working eight hours in the same chemical environment (Capurro, 1980). The individual working the longer shift would also have a greater chance for accidents in the work place, again due to fatigue and greater chemical exposure.

LIFE STYLE: Life style would consist of whether or not an individual possesses any of the following habits: 1) smoking cigarettes, 2) smoking pot, 3) take drugs such as amphetamine, cocaine, LSD, etc., 4) extensive alcohol consumption, 5) sniffing volatile agents, 6) overeats and thus is overweight, 7) is non-physical (does not exercise), 8) nutrition is extremely poor, 9) taking medications for various ailments, and 10) other factors

including stressful, worrisome and anxiety states of mind. The individual who can be characterized by one or more of these habits could be at a significantly greater risk than the individual who possesses none of these habits and is in excellent health. This is analogous to many disease states present in man such as atherosclerosis, lung cancer, and cancer in general. In the case of lung cancer, a smoker has a greater chance of lung and other organ cancer than the nonsmoker. Similarly, in atherosclerosis, the individual who eats foods with a lower content of cholesterol is at a lesser risk of developing atherosclerosis than the individual whose diet contains higher levels of cholesterol and saturated fats. In addition, an individual who works in chemical processing and related fields has a greater risk of cancer and other abnormalities than the individual working in a non-chemical occupation, e.g. office worker (Moszczynski, 1980); on the other hand, the office worker would be at a greater risk of heart disease related to such sedentary work.

RACE: A review of the literature with respect to toxicities of particular chemicals (solids and solvents) revealed that no differences in observed effects existed among reports from Europe, Asia, Russia, and the U.S.A. The commonality of effects suggests that no differences in response to toxicants were apparent among the races or various ethnic groups as populations. However, individuals among the races may exhibit differences dependent upon genetic and epigenetic traits.

SEX: There seems to be no difference in the relative amounts of chemical exposure in relationship to retention of chemicals by males or females. A study performed by Nomiyama and Nomiyama (1974) showed that the retention time of benzene following exposure was similar in each sex. After exposure to benzene, women had a greater blood concentration for the first 2 hours. Following the 2 hour period men and women converged to identical blood levels. There is some evidence suggesting that women may have a tendency to have a higher uptake and excretion of organic solvents than men. This may be attributed to the differences in adipose tissue and water content between men and women (Sato et al., 1972; Bartonicek, 1962). At this time there is not conclusive evidence confirming this suggestion.

GENETIC HISTORY: Individuals expressing toxic responses upon exposure to chemicals and solvents were once termed "hyper-sensitive" or "idiosyncratic" responses. Further studies revealed that these "individual susceptibilities" could be accounted for by the genetic makeup of such individuals, now recognized as having genetic variances e.g. glucose-6-phosphate dehydrogenase (G6PD) deficiency. Otherwise, healthy individuals

possessing this deficiency when exposed to aniline or aromatic nitro amine (nitrobenzene) derivatives would suffer erythrocytic damage and lysis resulting in hemolytic anemia, a life endangering condition. Therefore, these individuals having the G6PD trait must be identified and protected from such exposures. Several other enzyme deficiencies or variants can be recognized by pre-employment history and/or blood tests, e.g. atypical cholinesterases, slow/fast acetylators; endowing "susceptibility" to esters and hydrazine derivatives, respectively.

In summary, we have discussed several aspects of chemicals and solvents involved in exposures. We first discussed the interaction of a single agent with the body, followed by interaction of one or more agents. Secondly, we cited the literature regarding various host factors with relationship to solvent toxicity. Finally, we related how the physical and chemical characteristics can affect the mode of delivery of an agent to an individual and we attempted to demonstrate how these characteristics can affect solvent toxicity.

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OPEN FORUM IV

Dr. Slonim (AMRL): I want to ask a question of Dr. Bull about one of the last slides he showed on the mutagenic effects of acetonitrile. It seemed to me that when you were plotting mutations against dosage there was a fantastic rise at 2400 which instead of plateauing at 3600 reversed itself. Did you mention why that happened or would you do so now?

Dr. Bull (U. S. Environmental Protection Agency): What you are referring to is the initiation/promotion experiments in the Sinclair mouse. Yes, with dibromoacetonitrile it's a real effect. You get a very high response at the intermediate dose. It turns out that chemical is a very irritating material and what we suspect has happened is that it is cytotoxic and on the abraded skin it probably wipes out the initiated cells as well as the normal cells. That accounts for the fact that the response at low doses goes up quite high and then falls off which is a characteristic of the dibromoacetonitrile.

Dr. Couri (Ohio State University): Is it dehalogenated?

Dr. Bull: They probably are dehalogenated.

Dr. Couri: I mean at the high dose and because of that you may have a fall-off of activity.

Dr. Bull: No, I don't think so. These are topically applied.

Dr. Couri: I thought it was drinking water.

Dr. Bull: Yes, this is topically applied through the skin.

Dr. Slonim: That was a halogenated acetonitrile.

Dr. Bull: It was dibromoacetonitrile. The one that gave the response that you are referring to is trichloroacetonitrile which gives the same response but the dibromoacetonitrile is very irritating. At the high dose the mice actually had sores on their backs, and that's what I'm referring to. It's cytotoxic to the cells on the mouse's back and it's killing both initiated cells as well as normal cells. The reason for the fall-off of the dose response at the high end is cell death rather than mutation.

Col. MacNaughton (AMRL): Dr. Couri, would you please comment on why you think that the mice had no paralysis at all, even at very high concentrations of n-butyl ketone?

Dr. Couri: We are not sure why, because the definitive experiment has not been done. The one obvious possibility is that the mouse does not form the gamma diketone as a metabolite.

for that matter. I believe that a similar event happens with hexane exposed mice. It's probably a variation in metabolism of the parent compound.

Dr. Yang (NIEHS, NTP): I have a kind of philosophical question I would like to pose to the entire panel. At NTP we are interested in doing toxicological studies on a series of compounds which are identified as high priority hazardous chemicals in chemical dump sites. When you deal with a dump site situation or hazardous chemical dump site you are dealing with mixtures and it's not just two or three or fifty, it's hundreds or even thousands of different chemicals. If you have to deal with a situation like this and certain types of toxicological studies are to be done, as I can see it, you can approach this problem from two different viewpoints, both of which are extremes. One is that you use chemically defined mixtures, probably starting out with one and two and perhaps going up to three or four. The other approach is you go to the dump site to pick the sample or samples for testing. Both approaches are subject to criticism because the former one doesn't represent the real dump site and the latter problem, when you go to the dump site to sample, is how to collect a representative sample. Even if you have a representative sample for one site, does it constitute a representative sample for all dump sites? You can take this drum or that lagoon or whatever and come up with what you think is a representative sample. Suppose you were to take a chemically defined mixture. This afternoon I have not heard about more than two compounds in combination. There is one very interesting study published in the literature that was done in 1953 at NCI on 10 amino acids. They combined amino acids and then did a series of studies by removing one at a time and it made a very interesting study. When you go beyond two compounds, let's say 15, I calculated that, just using one dosage level, you divide the combination by the number of components and say $1/2$ for two combinations, $1/3$ for three combinations and for a mixture of 15 chemicals it requires 32,600 some groups for testing. The obvious difficulty is at this point. The dump site situation is a very dangerous one and very emotional and a very serious problem, because I understand half of the U.S. population is dependent on ground water or surface water. So I would like to pose this question to the distinguished panel. If you were to design some studies to approach this problem, not even thinking about solving it, what would you do?

Dr. Bull: I think there are some additional difficulties to the approach that you suggest of taking the sample from the dump. It is very rare that the exposure to humans has anything to do with the dump or the sample that is taken from the dump. You have to go another step further and say there is contamination of a ground water supply. That's what people are exposed to and I submit that the biggest problem you have is not trying to identify those chemicals, it's realizing that when the ground water is contaminated with trichloroethylene and tetrachloroethylene which are almost universally present in that kind of circumstance that

can be identified, you have more to worry about what can't be identified. Even in the most severely contaminated ground water situations where trichloroethylene and tetrachloroethylene concentrations are well up into the hundreds of parts per million range you would still be hard pressed to produce any toxic effect with those chemicals at that concentration in experimental animals. So, you are focusing on what you can see from an analytical standpoint and a very much more dilute kind of circumstance. To make any sense you have got to talk about the mixture that the people are exposed to, not what you see in the dump site. A dump site may have a barrel that leaks tomorrow and the next one leaks five years from now and they don't necessarily have the same thing in them.

Dr. MacFarland (Consultant in Toxicology): The dump site problem is just one example of this problem. There is difficulty in dealing with complex mixtures which are not compositionally fixed and incidentally that is why I gave all the information on the gasoline. That was only one gasoline. Gasolines vary all over the country. Let's consider the case of the simplest possible mixture. Two things and we've got 50% of each of them and we administer this and we get a certain effect. And then we take another sample of this mixture and we analyze it and we find this time we've got 51% of one of them and 49% of the other. The question that arises is there going to be a detectable biological difference in the action of that slightly different mixture? When we think about the sensitivity, or lack thereof, in our bioassay we would probably say that since we can't see that small a change as a practical matter we won't bother to test that. It's going to do the same thing as the true 50/50 mixture. Now we'll move this argument up a bit. We've got another batch of this stuff and this turns out to be 55% of one and 45% of the other. Are we going to have a difference now? We're getting into a kind of grey zone where maybe we will. If it's 70% of one and 30% of the other ingredient we would be almost certain that there will be a difference and we could probably detect a difference in the biological effect and we would have to do the experiment. This question comes up all the time with mixtures and there is simply no a priori answer to it. On the other hand, there are sometimes some common sense considerations that give you a practical answer. If the difference only turns out to be 1% you probably wouldn't fuss about it and if the difference turned out to be tremendous you probably would. There is a great big grey zone in the middle where you don't know what to do and if you've got the money and the time you do the experiment. We often look at these things and approach them theoretically, and we face just what you did. You get these two extreme positions on the thing and you look at both of them and they are both impossible. You know in the real world that we don't usually enter into these problems in a state of total ignorance and often we can make some sort of simplifying assumptions and we can make an intelligent selection of certain mixtures to look at on the basis of assorted information that we have. Another thing that

bothers me a great deal when speaking of these dump site mixtures is that I think we've got to throw out this concept of a "representative mixture." There isn't such an animal as a representative mixture if you are dealing with something that is apt to have one composition today and an entirely different one tomorrow and which when it's moved downstream is something entirely different again. So you know a representative sample is only applicable when the range of compositional variation is not too great.

Dr. Couri: Dr. Abdel-Rahman, there is probably more than one dump site in New Jersey and you might have an appropriate comment.

Dr. Abdel-Rahman (New Jersey Medical School): I had a case two years ago of one couple came to me because the wife had one infection after another and then get another infection. They said that the water smelled bad. When I asked where they lived, they said that they lived in a very nice resort area in Pennsylvania. When I asked about their water source they told me they drank well water. They were supposed to have good well water. I recommended they have the water analyzed and they did and they came back to me with a big list of contaminants including three thousand parts per million carbon tetrachloride, two thousand ppm chloroform, and some bromodichloromethane. Every chemical compound you hear about as being carcinogenic was present in the water. What was going on out there? I couldn't believe that this was really well water. We studied the situation further and we found that one year earlier an explosion had happened in a gas station located 500 feet from their home and the tank of gas leached into all of area. His well water was contaminated. I told him the first thing to do was to get a new source of water even if you have to move away from this home, because the water you have is so bad that even if you use any kind of a charcoal or any kind of filter it will be saturated within one day. In such a case as this we have to go to the literature to see what is the health effect of each of the chemical pollutants and as a toxicologist I don't think we can really design an experimental study of hundreds or tens together. I don't know who can afford to do that either physically or financially. You saw today that when we have one chemical, two chemicals, or even three chemicals we are in good shape to conduct studies. Someday we may understand the health effect of this combination mixture but not today. The best advice I could give this person when they came was to try charcoal filters and even then we would like to check the water every week. He listened and he went and put in a charcoal filter and it has decreased the pollution very well. However, he forgot to change the filter one week and the same concentrations of pollutants came back. So I told him not to use it for drinking. If they used it even for washing it would have to be heated and maybe the high temperature would volatilize out these kind of things. At the very least they should avoid ingestion of this material.

Dr. Couri: If there is anyone in the audience that would like to comment on Dr. Yang's question? I think that it bears on everyone and there may be some ideas that you would like to present.

Dr. Holdsworth (American Petroleum Institute): Dr. Yang, as Dr. MacFarland intimated in his comments, at API we deal with that problem every day in designing studies and it's not a very easy question to answer. I think the thing that you are conveying is the difficulty of selecting what to test and you can look at all the scenarios in the world and still not find a representative sample. You may have to pick a typical or several typical samples to look at and test. I don't know how many of you picked that point up in Dr. MacFarland's talk but he is absolutely right. You just can't predict a result when you have hundreds or thousands of compounds in a complex mixture. You've got to do the test. It becomes a real problem when you want to be cost effective with your research. Where do you place the research dollars? We're facing this problem with ground water contamination right now. We are trying to get an idea from EPA and from our own people, about what kind of contamination of ground water we're dealing with so that we can design some kind of a chronic study. It's a mind-boggling problem. All of us share that concern. While I've got the floor, I'd just like to comment on something else. In reference to the gasoline study, our best evidence right now is that primary response of rat nephropathy is due to the isoparaffins in the mixture. We're pretty certain from the weight of the evidence that it is not caused by benzene or aromatics at all, even though the gasoline contained 2% benzene. We don't see any effects of benzene in a gasoline study nor do we see the effects that you would anticipate from benzene itself. In other words, you don't see hematologic effects from chronic exposure to gasoline and the kidney tumors are not caused by benzene. One other thing that I'd like to comment on is that we have been screening a number of compounds in gasoline to try and come down to structural activity relationships relative to nephrotoxicity at least and this group might be interested to know that n-hexane is also nephrotoxic. We think there is a definite structure activity relationship. We don't know how broad or how narrow it is. We have tested a number of compounds. As Dr. MacFarland indicated, it is the isoparaffins that seem to be biologically active. The other classes such as olefins and aromatics seemed not to be active. The point is, we don't know how those other compounds affect the activity of those nephrotoxic chemicals in gasoline. It's going to take a lot of work and a lot of effort to work that out and we're trying to do that now. This toxic effect occurs only in male rats as far as we know. We've looked at these solvents in a number of other species, both in mixture and individual compounds and don't see the nephrotoxicity or the nephrocarcinogenicity.

Dr. Torkelson (Dow Chemical): In this room of gloom and doom I hate to be a little bit optimistic but from what I've heard today are we really talking about much more than additive

toxicity? I haven't heard a great deal about potentiation or much more than you would predict from simple addition and I guess that it is at this point when we have potentiation that I would get concerned.

Dr. MacFarland: I don't think you're right, Dr. Torkelson. You remember that I made some remark to the effect that while synergistic and antagonistic effects are of the greatest interest, those are of infrequent occurrence. I think most of the speakers today, however, were emphasizing the infrequent occurrences. Most of them were talking about one form of interaction or another.

Dr. Bull: I don't think you could classify what I have discussed as additive effects. I'm not sure what I would call that but when you are talking about a reaction of chlorine with an amino acid to produce a heteroacetoneitrile I'm not sure if you are dealing with additive synergistic toxicity because you have a different product. Obviously amino acids don't have an adverse effect in their own right and it's only the reaction product that does.

Dr. Torkelson: It's the mixtures of chemicals that are actually being given. You made a new chemical by chlorinating it but now is that mixture any greater than the sum of its parts?

Dr. Couri: If I could comment on the data I have shown. I don't think I used the word synergism, but if I did I'd stick by that definition. I think the action is synergistic. The table was entitled as enhanced toxicity of methylethylketone on the effect of methylbutylketone. In one experiment I showed that six out of six animals exposed to the mixture died and the positive controls exposed for nearly three times as long at twice the concentration of the individual components showed no effect. Would you not agree that is synergistic? Exposure to four hundred parts per million of methylbutylketone for 60 days gave two mildly crippled animals out of six. Two hundred twenty five parts per million in the presence of the second solvent was lethal. Isn't that a synergistic response?

Dr. Torkelson: I think that my statement is correct that most of the data that has been presented showed additive effects but whether it is synergism or additive gets to be hairy. However, most of the discussion I've heard today was, I think, closer to describing additive effects than it was to potentiation.

Dr. Couri: Would you tell us what difference it makes whether it is additive or synergistic?

Dr. Torkelson: Well, I think it simplifies your problem.

Dr. MacFarland: Dr. Torkelson, suppose you have subjects A & B and you're experimenting with the LD50 and the LD50 for A is 100 units per kilogram and for B it's 25. Now if you administer 25 units of B you get a certain endpoint. If you administer a 100 units of A you get the same endpoint. All right now, let's imagine that we do this experiment. We take 50 of A and we take 12.5 of B as a mixture and we administer that and we get the same endpoint. If that happens you are dealing with a simple, additive action. That's an addition. How many of the examples did you hear today that would fit that? Scarcely one.

Dr. Abdel-Rahman: Can I make a comment? In the teratogenic study of malathion and carbaryl there was really clear evidence of much greater effects of the mixture than with either one alone. With a mixture of 1 mg/kg carbaryl and 1 mg/kg malathion you find fetal resorption but even with 100 mg/kg of either no fetal resorption occurred. There is really no question about this. It is a synergistic effect.

Dr. Holdsworth: I certainly don't want to be all gloom and doom because I'll tell you there are a lot of interactions that show just the opposite. You see a decreased effect. Dr. Couri mentioned the influence of toluene on benzene. I think it depends on the endpoint you look at. There is evidence to show just the opposite, that toluene suppresses the action of benzene and you know we talk about these things a lot of times.

Dr. Couri: In all fairness, I think Dr. Andersen's presentation showed four fine examples where there was nullification if you will or indication of the interaction. You're referring to the metabolism of toluene/benzene as competitive substrates in an oxidative system. I would agree with that. Those are fairly well studied combinations, given in such a way as to produce less of an effect of benzene. You can reverse that easily just by reversing the ratios of toluene to benzene administration. I would agree with you that those interactions can go either way. Greater, less and perhaps no change. As matter of fact three alternatives. Dr. Andersen, do you want to mention your study?

Dr. Andersen (AMRL): I didn't go out of my way to pick inhibitory interactions but I do think there are a lot of inhibitory interactions. Especially in instances where you are dealing with a metabolite. I agree with Dr. Torkelson that most of the interactions you deal with will be either additive or less than additive. That will be the run-of-the-mill response, but when those synergistic ones come along they have a great impact. They have an impact on our science because they force us to go out and find out what is going on. They also have an impact on the public because the public thinks that all these chemicals are very bad and that two of them together are very much worse. The public's information is not on the inhibitory interactions. It's on such things as the huffer and puffer syndrome in Miami. I don't know how to predict synergism. When they occur they are

remarkable reactions and demand a lot of attention from us to explain what's going on and to understand the underlying mechanisms that drive the synergism so that we can say that there are certain mixtures that we are going to have to avoid. Right now synergism is very difficult to predict.

Dr. Miller (Ohio State University): I want to continue the answer to Dr. Yang's question. We have to realize that it's partly a matter of education. He said that it's an emotional problem and I think that that hit the nail on the head. We must educate the public to the fact that our bodies require small amounts of most things and even the most innocuous substance will kill us if we get too much of it. Now one other comment. Dr. Abdel-Rahman mentioned the man who had hundreds of things in his drinking water. What can you do? Well, reverse osmosis water is a partial answer. This is commercially available. My wife happens to be a plant nut so we have reverse osmosis water in our home for the plants and of course it's of some benefit to us. It's not very expensive.

SESSION V

**IMPACT OF TOXICOLOGIC INFORMATION
ON OCCUPATIONAL HEALTH MANAGEMENT SYSTEMS**

Chairman

**B. Dwight Culver, M.D.
University of California
Irvine, California**

**AN UPDATE ON THE CAPABILITIES OF THE AIR FORCE COMPUTERIZED
OCCUPATIONAL HEALTH PROGRAM (COHP)**

Maj. C. Donald Worthy, Jr., USAF, BSC and
Karen A. Meier, LTC, USAF, NC

Brooks Air Force Base, Texas

The Air Force has been conducting an aggressive occupational health program for over 30 years. The ultimate goal of the program is to protect the worker--both military and civilian--by providing a work environment that is free of recognized chemical, physical, or biological health risks. The success of the program has been and will be an essential contribution to our country's overall military readiness.

The increasing complexities of managing occupational health programs fueled by recent legal Department of Defense mandates, escalating costs of workers' compensation, influx of more complex chemical compounds in the work place, and public awareness of environmental pollution have prompted the Air Force to develop innovative approaches to the field of occupational health. In May of 1983, our Surgeon General directed the Air Force's occupational health program to be managed under the umbrella of the Comprehensive Occupational Health Surveillance Program (COHSP). The COHSP program consists of six subprograms (Table 1): the Standardized Occupational Health Program (SOHP) manually standardizes and links the data input for the worker and the workplace exposure; the computerized program (COHP) automates the manually generated data base; the DETECT program provides the capability to evaluate and select the best sampling procedures and instrumentation to meet the needs of the Air Force; and PROTECT provides the capability to evaluate and select the most appropriate personal protective equipment to meet the needs of the Air Force worker. The last two subprograms, TRENDS and COMPARES, provide the mechanism to evaluate the human effects of exposure to hazardous chemicals, noise, and radiation. For instance, human exposure data could be compared with animal toxicologic studies to increase our knowledge in man's responses to the effects of chemical, radiation, and noise agents.

**TABLE 1. COMPREHENSIVE OCCUPATIONAL HEALTH
SURVEILLANCE PROGRAMS**

Subprogram Elements

SOHP/COHP: Standardized/Computerized Occupational
Health Program

DETECT/PROTECT: Detection/Protection Equipment
Capabilities and Technologies

TRENDS/COMPARES: Toxicology Research and Epidemiology
of Noise and Dangerous Substances/Computerized
Assessment of Radiation Exposures

SOHP/COHP and DETECT/PROTECT are operational programs that will be managed at the USAF Occupational and Environmental Health Laboratory (Table 2). TRENDS and COMPARES are follow-on epidemiology and toxicology programs that will be managed by both the Air Force Aerospace Medical Research Laboratory and the Air Force School of Aerospace Medicine, respectively. Today the presentation will be limited to two areas, SOHP and COHP.

**TABLE 2. COMPREHENSIVE OCCUPATIONAL HEALTH
SURVEILLANCE PROGRAM MANAGEMENT**

USAF OEHL Program Office
SOHP/COHP
DETECT/PROTECT

AFAMRL - TRENDS

USAFSAM - COMPARES

The main contribution of the Standardized Occupational Health Program was to reorient and standardize the method we use to collect and store program related data in the Air Force. Our purpose was to rapidly retrieve these data and ensure a linking mechanism between the worker and the workplace exposure. To accomplish this mechanism we created a Workplace Identifier which is to the workplace as the Social Security Number is to the worker. In spite of the improvements, SOHP is a manual program. It is admittedly cumbersome and labor intensive. Thus, it hinders timely applications of the data at all levels of the Air

Force including the research community. Management studies were conducted in 1981 to evaluate the feasibility of computerizing the Air Force's occupational health program. An Air Force Management Evaluation Team concluded that computerization was feasible and cost effective.

The Computerized Occupational Health Program (COHP) will satisfy the needs of many diversified customers ranging from base level bioenvironmental engineers, environmental health personnel, and health care providers to research scientists. The key requirement for the primary users is real-time access to data. The system must be capable of displaying worker/workplace data instantly, if the information is to be useful in health care decision making. From a management viewpoint, COHP must feature an occupational health information system that has current summaries of workload discrepancies and exposure data to determine the status of operating programs and enhance policy decision capability. Existing automated data bases, such as our hearing conservation registry and National Institute for Occupational Safety and Health (NIOSH) Registry of Toxic Effects of Chemical Substances, must be networked with COHP to effectively utilize all available data, both past and present. Comprehensive statistically accurate data must be available to enable support and research laboratories to obtain necessary information from the operational activities.

Interwoven with these features is the design specification that the system be user friendly, that is, the system does not require computer specialists but can be handled by anyone who possesses basic typing skills, is familiar with multiple choice test questions and is able to fill in correct data in the appropriate blanks of a form. COHP data will be entered by the person most familiar with the way in which the data were obtained. In other words, the person who collects the data is the one who enters the data. This will not only cut manpower costs, but will improve data accuracy.

As you can see from the following computer demonstration, there are many places in the program that the user encounters what we call "applied toxicology." As the data base builds over a period of time, it will provide increased capability to contribute to epidemiology and toxicology studies.

TABLE 3. MENU SCREEN

COMPUTERIZED OCCUPATIONAL HEALTH PROGRAM

MASTER MENU

1-INDUSTRIAL HYGIENE PROGRAMS
2-CLINICAL OCCUPATIONAL HEALTH PRGM
3-ENVIRONMENTAL PROTECTION PROGRAMS
4-MANAGEMENT
5-EMERGENCY/ACCIDENT ASSISTANCE

SELECT MENU ITEM(0-EXIT):

TABLE 4. DATA REVIEW SCREEN

WORKPLACE OPERATIONAL DATA

DATE: 8104 WORKPLACE ID:0163-FACC-005A ORGANIZATION:12 FMS/MAFFC
NAME: CORROSION CONTROL BUILDING NO: H. 48 ROOM/AREA : HANGER

ADMINISTRATIVE

1-SUPERVISOR: GEORGE S. HART 2-DUTY PHONE: 6965 3-OFFICE SYM: 12MANF
4-FAC: 5-OSC: 6-SHIFTS/DAY: 2 7-DAYS/WEEK: 5

NARRATIVE DESCRIPTION

1- MODELS AND MOCKUPS FABRICATED IN SHEETMETAL. POWERTOOLS FOR CUTTING
2- AND SHAPING SHEETMETAL. HYDRAULIC TEST STAND IN NORTH EAST CORNER.
3- SANDBLASTING BOOTH AND VAPOR DEGREASER FOR STRIPPING OLD PARTS.
4- SAWS, DRILLS, RIVETER, AND CUTTER USED BY EVERYONE. WORKTABLES FOR
5- SHAPING & ASSEMBLY. SOLVENT TANK USED FOR HAND WASHING OF PARTS.

TABLE 5. EXAMPLE OF INVERSE VIDEO TO FLAG PROBLEMS

OCCUPATIONAL HEALTH WORKPLACE EXPOSURE DATA

DATE: 8104 WORKPLACE ID:0163-FACC-005A ORGANIZATION:12 FMS/MAFFC
 NAME: CORROSION CONTROL BUILDING NO: H. 48 ROOM/AREA : HANGER

FOR MONTH OF: JAN 1983

DATE	SOURCE	RESULT	WORKER	S-TYPE	CONTROL
1-821202	NOISE--HYD. TEST STAND	81 dBA	REP	TWA	EAR PLUGS
2-821203	PD-680 PART CLEANING	10 MG/CM	MAX	STEL	RUBBER GLOVES DILUTION VENT.
3-830126	1,1,1-TRICHLOROETHANE	1155 MG/CM	REP	TWA	LOCAL EXHAUST WATER JACKET ORG.VAP.RESPIR

PAGE 1 OF 1

SELECT OPERATION [EXIT] : -- 1- REVIEW WORKPLACE DATA
 2- REVIEW RECOMMENDED EXAMINATIONS

TABLE 6. HAZARD EVALUATION DECISION

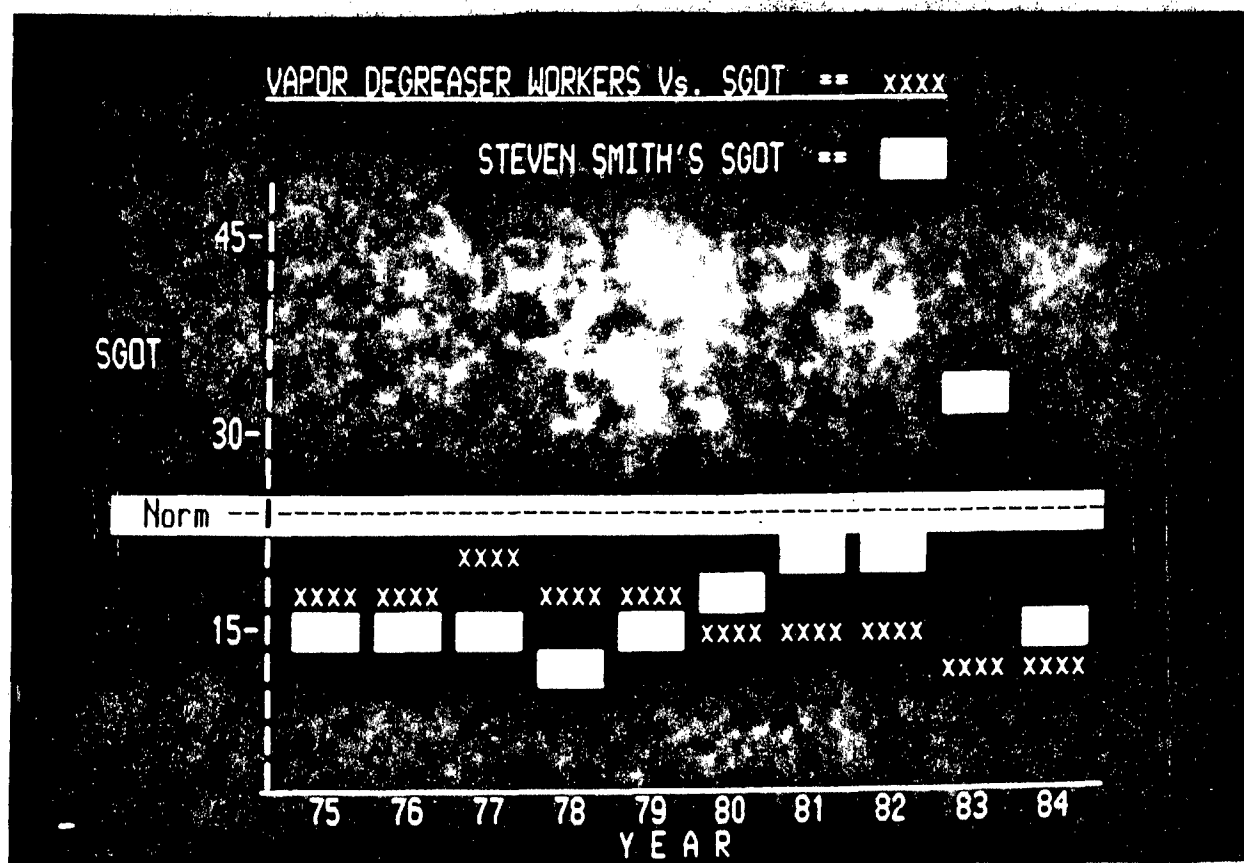
EXPOSURE CONFIRMATION

CASE FILE #: 0058 ORG.: 12FMS NAME: CORROSION CONTROL

MATERIAL COMPONENT	MSDS?	QUANT USED PERCENT	IEX	POT/ACT INH ABS	HAZARD ING CON
TRI-ETHANE	YES	40 GL/MO	8		
1,1,1-TRICHLOROETHANE		100 %		yY n	y y
-----		%			
-----		%			
-----		%			
-----		%			

>>> IS THIS POTENTIAL HAZARD POSSIBLE [NO]? -- 1-YES

TABLE 7. BIOLOGICAL SCREENING TREND ANALYSIS



In summary, SOHP is the basic building block for the other subprograms (Figure 1).

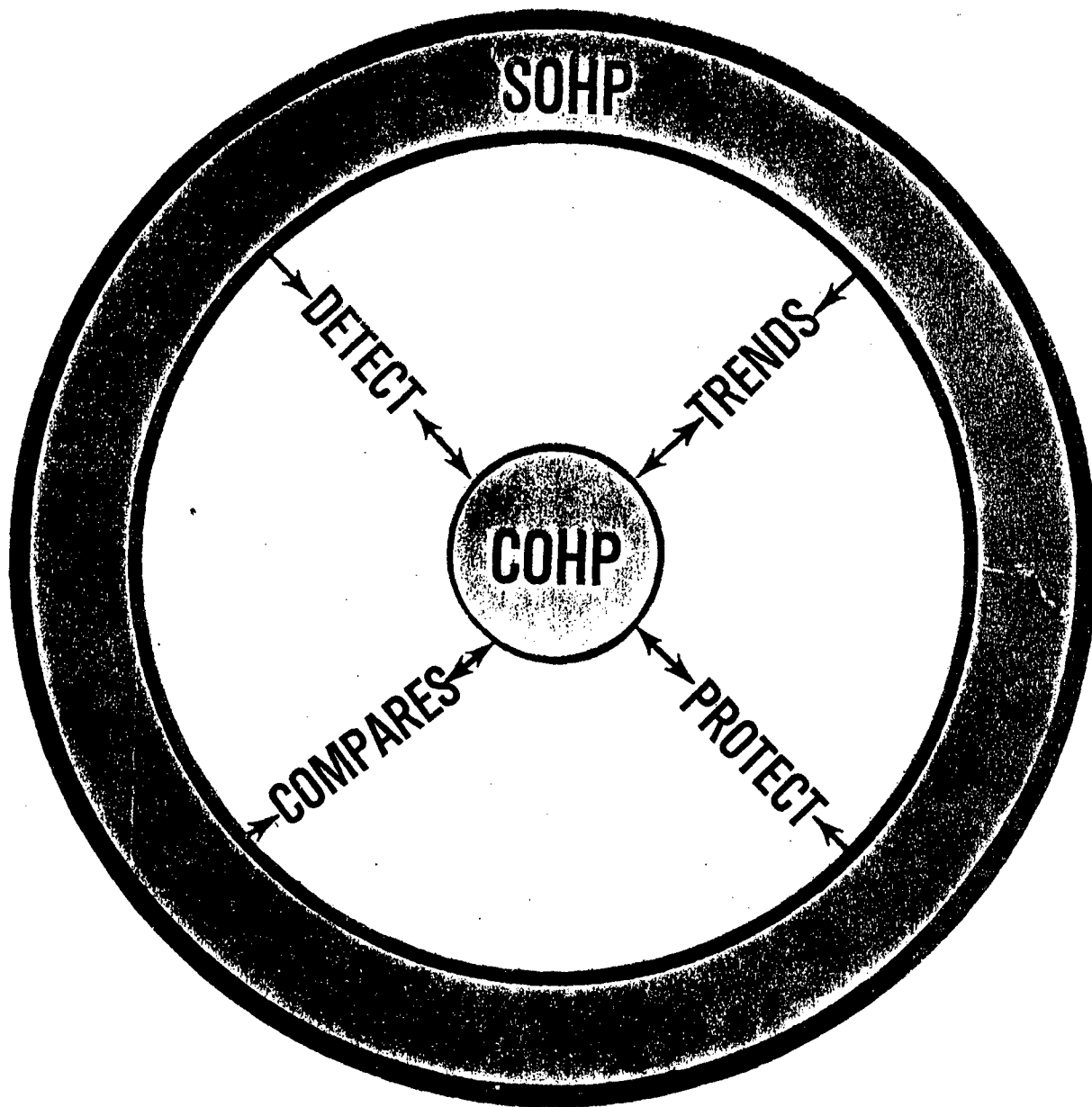


Figure 1.

COHP is the nucleus of the Comprehensive Occupational Health Surveillance. Without computerization, COHSP loses its interactive potential to conduct effective, detailed epidemiological and toxicological research.

THE EPIDEMIOLOGY AND TOXICOLOGY OF AGENT ORANGE

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VIETNAM BACKGROUND

An estimated 107 million pounds of herbicides were aeri-ally-disseminated on three million acres in South Vietnam from January 1962 through October 1971. Approximately 94 percent of all herbicides sprayed in Vietnam were 2,4-D (56 million pounds or 53 percent of total) or 2,4,5-T (44 million pounds or 41 percent of total). The 44 million pounds of 2,4,5-T contained an estimated 368 pounds of the toxic contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). As most of you are probably aware, the phenoxy group of herbicides were developed in the mid-1940's and were extensively used in agriculture. In 1950, more than ten million pounds of these materials were used annually for weed and brush control in the United States. By 1960, more than 36 million pounds were used annually.

As we became involved in combat operations in Vietnam, the risk to our troops from ambush increased and military commanders, including General Westmoreland, asked that herbicides and the agricultural chemical technology be applied to the jungles in an attempt to clear areas around camps, roads, and other lines of communication. Their goal was to provide a clear field of fire for our troops and deprive the enemy of hiding places. In May 1961, the Secretary of Defense directed a program to test the feasibility of defoliating jungle vegetation in the Republic of Vietnam. By early fall, 1961, eighteen different aerial spray tests (defoliation and anticrop) had been conducted with various formulations of commercially-available herbicides. The choice of these herbicides was based upon the chemicals that had had considerable research, proven performance, and practical background at that period in time. Also, such factors as availability in large quantity, costs, and known or accepted safety in regard to their toxicity to humans and animals were considered. The results of these tests were that significant defoliation could be obtained with two different mixtures of herbicides. The first was a mixture of the n-butyl esters of 2,4-D and 2,4,5-T and the iso-butyl ester of 2,4,5-T. This mixture was code-named "Purple". The second "military" herbicide was code-named "Blue" and consisted of the acid and sodium salt of cacodylic acid. The

colored bands which were painted around the center of the 55-gallon drums served as aid to the identification by support personnel to avoid the mixing of incompatible chemicals by personnel unfamiliar with technical/scientific terminology.

After the preliminary testing was completed, three C-123 aircraft were prepared to apply the herbicides in January 1962 under the code name OPERATION RANCH HAND. The first shipment of Herbicides Purple and Blue arrived in the Republic of Vietnam on 9 January 1962. These were the first military herbicides used in OPERATION RANCH HAND, the military project for the aerial spraying of herbicides in South Vietnam. Two additional phenoxy herbicide formulations were received in limited quantities in South Vietnam and evaluated during the first two years of OPERATION RANCH HAND. These were code-named Pink and Green. By January 1965, two additional military herbicides, code-named Orange and White, had been evaluated and brought into the spray program. Herbicide Orange replaced all uses of Purple, Pink, or Green, and eventually became the most widely used military herbicide in South Vietnam. Purple, Orange, Pink, or Green, were all 2,4,5-T containing herbicides; White contained 2,4-D and Picloram; and Blue was cacodylic acid, a rapidly acting arsenical.

As the war intensified after 1965, the use of herbicides also increased. By 1968, thirty-six C-123 aircraft were participating in the herbicide delivery missions. Orange, White, and Blue were all used during these years, but the media and many of the veterans groups have tended to refer to all of these herbicides as "Agent Orange".

Herbicide Orange was the most extensively used herbicide in South Vietnam. Orange accounted for approximately 10.7 million gallons of the total 17.7 million gallons of herbicide used. It was used from mid-1965 to June 1970. However, Orange was not the only 2,4,5-T containing herbicide used in the defoliation program. Small quantities of Purple, Pink, and Green, all containing 2,4,5-T were used from 1962 through mid-1965.

Each of the three major herbicides (Orange, White, and Blue) had specific uses. Ninety-nine percent of Herbicide White was applied in defoliation missions. It was not recommended for use on crops because of the persistence of Picloram in soils. Because the herbicidal action on woody plants was usually slow, full defoliation did not occur for several months after spray application. Thus, it was an ideal herbicide for use in the inland forests in areas where defoliation was not immediately required, but where it did occur it would persist longer than if the area were sprayed with Orange or Blue.

Herbicide Blue was the herbicide of choice for crop destruction missions involving cereal or grain crops. Approximately 50 percent of all Blue was used in crop destruction missions

in remote or enemy controlled areas with the remainder being used as a contact herbicide for control of grasses around base perimeters.

Ninety percent of all Herbicide Orange was used for forest defoliation and it was especially effective in defoliating mangrove forests. Eight percent of Herbicide Orange was used in the destruction of broadleaf crops (beans, peanuts, ramie, and root or tuber crops). The remaining two percent was used around base perimeters, cache sites, waterways, and communication lines.

In addition to the herbicides, numerous other chemicals were used in South Vietnam. These included selected fuel additives, cleaning solvents, cooking oils, and a variety of other pesticides. The insecticide Malathion was widely used for control of malaria-carrying mosquitoes and at least 400,000 gallons of it were used from 1966 through 1970. In some areas of Vietnam, the illness rate for malaria was as high as 600/1,000/year. In addition, much smaller quantities of Lindane and DDT were used in ground operations.

Numerous aircraft were used in the air war in Vietnam, but only a few of these aircraft were used for aerial dissemination of herbicides. The "work horse" of OPERATION RANCH HAND was the C-123/UC-123, "Provider". This cargo aircraft was adapted to receive a modular spray system for internal carriage. The module consisted of a 1,000-gallon tank, pump, and engine which were all mounted on a frame pallet. An operator's console was an integral part of the unit, but was not mounted on the pallet. Wing booms (1.5 inches in diameter, 22 feet long) extended from the outboard engine nacelles toward the wing tips. A short tail boom (three inches in diameter, 20 feet long) was positioned centrally near the aft cargo door. Each aircraft normally had a crew of three men: the pilot, co-pilot (navigator), and flight engineer (console operator). During a typical mission, the C-123 sprayed at a speed of 150 miles per hour at a height of 150 feet above the ground, often at tree top level in the triple canopy jungle. On the average, each plane was hit 50 times per mission by ground fire.

Ten to 12 percent of all herbicides used in Vietnam were applied by helicopter or by ground application techniques (back packs or buffalo turbines).

Prior to 1970, studies in industrial groups had implicated trichlorophenol, a phenoxy herbicide precursor, as causing health problems, but no problems were recognized in the application of the final product. In 1970, however, 2,4,5-T was found to cause birth defects in laboratory rats and the Department of Defense stopped the use of 2,4,5-T herbicides in early 1970. The spray operation continued into 1971 using the other herbicides and ceased entirely in October of that year.

MEDICAL EFFECTS

While the phenoxy herbicides and TCDD have been studied extensively, clearcut information concerning the adverse effects of these chemical compounds in humans is extremely difficult to find. Acute and sub-acute effects are fairly uniformly reported following accidental excessive exposures, suicidal gestures, and industrial accidents. Yet there is a great deal of confusion concerning the presence or absence of truly long-term effects. Much of the medical knowledge concerning the effects of 2,4-D; 2,4,5-T; and TCDD exposures in humans is derived from individual case reports. Since many of the patients described in these reports were exposed to multiple chemical agents, it is difficult to determine which specific symptoms were caused by which chemical. Nevertheless, skin and nervous system diseases have been reported in many of these cases. A vast array of signs and symptoms have been attributed to 2,4-D and the ones reported most consistently include: nervous system, liver, psychological, and intestinal problems.

Since 2,4,5-T is contaminated with TCDD in the manufacturing process, its effects on humans are extremely difficult to evaluate. In all studies of 2,4,5-T exposures, its effects could not clearly be distinguished from the possible effects of TCDD. Symptoms attributable to exposure to 2,4,5-T and TCDD include all of the symptoms of 2,4-D exposure, plus the skin disease, Chloracne. Chloracne generally begins around the eyes and temples and is often found on, in, and behind the ear. It may result in extensive scarring and has been associated with premature aging of involved skin areas. In severe acute cases, lesions have spread to the throat, neck, axillary, and inguinal areas. The condition usually clears up spontaneously but has been found to occur periodically for up to 30 years after massive industrial exposures. Numerous epidemiologic studies of industrial populations have strengthened the link between exposure to TCDD and the development of Chloracne and associations between TCDD and psychological abnormalities have also been suggested. A series of Swedish studies found a link between cancer and exposure; however, it is difficult to make a cause and effect conclusion from these data. Other studies in this country and abroad have been conducted, and none has been able to provide firm evidence to answer the question of whether or not these exposures cause long-term health problems.

Currently, there are no epidemiologic data associating TCDD with any long-term health effects in humans other than intermittent Chloracne; however, while there is no evidence validating serious long-term health effects, neither is there strong evidence for lack of effect. Most previous studies have not had sufficient numbers of subjects to detect increased risks of uncommon conditions, and the period of observation in many studies has been inadequate to detect conditions with long lag

times between exposure and illness. There is currently no good evidence linking exposure to dioxin to cancer or birth defects in humans.

The U. S. Air Force released the first mortality report of its Ranch Hand (Agent Orange) study, an epidemiological investigation of the possible adverse health effects from herbicide exposure of Air Force members who conducted aerial spraying missions in Southeast Asia (Operation Ranch Hand). The purpose of the study is to determine whether long-term adverse health effects exist and whether they can be attributed to occupational exposure to herbicides and their contaminants.

The mortality analyses have not revealed any statistical excess in the deaths recorded in the herbicide/dioxin-exposed group. At this time, there is no indication that Operation Ranch Hand personnel have experienced any increased mortality or any unusual patterns of death in time or by cause. They are not dying in increased numbers, at earlier ages, or by unexpected causes.

Twelve hundred sixty-nine individuals who were assigned to Air Force units directly involved in Operation Ranch Hand were identified through extensive searches of military historical and personnel records. Most of these men were exposed to herbicides for up to 10-12 hours a day, five to six days a week, for periods of at least one year. Thus, the Air Force considers them to be the most heavily herbicide-exposed group of U. S. military personnel in Southeast Asia.

For the purposes of statistical comparison, a group of other Air Force personnel assigned to duty in Southeast Asia were matched to the Ranch Hand group by race, job, and date of birth. Five comparison subjects were selected for each Ranch Hand to improve the ability of the study to detect a difference in the death experience. The death experience of the herbicide/dioxin-exposed group was contrasted with the death experience of the matched group, as well as with three other groups external to the study: the 1978 U. S. white male population, a Department of Defense (DOD) retired population, and the 1956 graduating class of West Point.

Analyses showed that, to date, the mortality experience of the Ranch Handers is identical to that of the matched comparison group. The overall rates and causes of death are not significantly different between the groups. However, it does appear that officers are living longer than enlisted personnel in both groups.

The Ranch Handers did show a relative decrease in cancer, but an increase in liver disorder deaths; however, both these findings are statistically nonsignificant. There were no soft tissue sarcoma deaths diagnosed in either group.

Analyses of both the Ranch Hand and comparison groups to the 1978 U. S. white male mortality experience showed highly significant findings of lower mortality rates among Air Force members. Most of these differences can be attributed to the healthy worker effect (due to the fact that individuals must be in good health upon entry into the military, while the general U. S. population is not subject to this form of "selection").

The DOD retired and West Point data were used for further analyses in an attempt to correct for the healthy worker effect. The contrast with the DOD data again demonstrated significantly less mortality for Ranch Hand officers, comparison officers and comparison enlisted men; however, there was not a statistically significant favorable mortality rate for Ranch Hand enlisted personnel. The West Point comparison had to be restricted to an analysis of the officer groups since all West Point graduates become officers. This analysis revealed no differences in mortality.

The fact that adverse effects have not yet been detected does not imply that an effect may not become manifest in the future. For this reason, further analyses are intended and mortality in the study population will be ascertained annually up to the next 20 years.

WHAT WILL THE FUTURE BRING?

We can be sure of several things over the next few years. As in the past, social policy will be made despite scientific uncertainty and the lack of definitive answers to the complex questions posed by exposure to chemical and physical agents.

Environmental consciousness will likely increase in all segments of our society. This has become a "quality of life" issue, and as such, will be an important issue to many people.

There will be a continuing trend to guarantee a "risk-free society" or at least a society in which the only risks we are exposed to are voluntary ones, e.g., cigarette smoking, over-eating, exceeding the 55 mile per hour speed limit, failure to wear seatbelts or motorcycle helmets, mountain climbing, etc. Self-imposed risks will be acceptable; involuntary risks will not be acceptable.

Fear of a "cancer epidemic" will continue. In the absence of scientific data which are presented in a manner that is easily understood by a non-technical or non-scientific public, this fear will remain a highly emotional issue in the minds of many.

As the concern for the environment and health risks increase, the use of more and more chemical and physical agents will be questioned. We have seen it with DDT, saccharin, 2,4,5-T, cyclamates, nitrites, and food preservatives. 2,4-D, microwave radiation, and nuclear power are some of the current issues, and many others should arise in the future.

WHAT CAN WE DO?

In the past, emotionalism has played a major role in decision-making. When we as scientists fail to explain our work in terms that lay people can understand or in ways which appeal to the media, our evidence is often misrepresented or ignored by the media, the public, and the social decision-makers. We should strive to present factual material to the media and the public in a manner that is understandable. We must avoid jargon and highly technical explanations. Our goal should be to provide accurate information in an unbiased manner. If our results indicate that a problem exists, we must present them regardless of the economic effect. Facts presented in a clear and understandable manner will be publicized by the responsible media, and unreasonable fear based on emotion and misinformation will be alleviated. We must correct factual errors in information provided to the public and stress the beneficial aspects of modern technology.

EARLY DETECTION OF ENVIRONMENTAL EXPOSURE

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Thank you very much for that introduction and it really does give me a sense of "deja vu" to be on the same program with Dr. Culver after all these years, still talking about some of the same types of problems that we recognized when we first worked together out in the San Bernardino, Los Angeles area when I was associated with the development of the Ballistics Systems Division, Occupational Health and Safety office there, and when Dr. Culver was the Medical Director of Aerojet General. As I was recalling that association I was reminded that having just completed full residency training in occupational medicine at that time I was very eager to apply what I had learned in the practical situation. Actually I finished Kettering in 1956 and from 1956 to 1959, I worked at the headquarters of the Logistics Command. At that time it was called the Air Material Command. I attempted to develop improved standards of data collection on occupational and environmental health at that time. But the time was not right then and there were many many types of inertia present that made it very difficult to mount a program from headquarters and when General Wilford Hall, who incidentally is the person that Wilford Hall Medical Center is named for, came to be the new command surgeon there at Wright Patterson he said that he was convinced that what we really needed was to have a program at base level that could deal with some of the practical problems and be a basis for then setting up a model that could be extended more widely. It was with that in mind that I went out to Norton and because of some of the staffing problems I found it took me a while to get started because we were confronted with having to run a hospital as well as an occupational medical facility and for awhile there I was Commander of the hospital as well as head of aerospace medicine which included occupational medicine and it was just very difficult to have an extremely well worked out program there. In the midst of all of that was the Ballistic Systems Division which is one of the biggest construction projects that our country has had since the railroad was initiated. It became quite apparent that certain types of toxicological information were needed in order to direct our handling of some of the new weapons like the Titan II propellants. I can remember

one symposium that we held at Englewood, at Ballistics Systems Division to try to answer questions such as this. If you had a disaster and you had a major spill of propellants from Titan II and you had hundreds of people coming out of the cloud, what would you do to try to be sure that any illness from that exposure was promptly and properly recognized and treated in order to save lives and prevent morbidity? We had quite a wide range of opinions from internists and pulmonary specialists and scientists of different types, toxicologists and on the one extreme, one medical director said, why since they have the possibility of developing the often fatal complication, bronchiolitis obliterans fibrosa, I would put them all on steroids and at the other extreme, Dr. George Wright, whom some of you know, said well there you would have the problem of introducing the possible side effects of the steroids with perhaps an even greater health threat. So eventually a position was worked out whereby all would be observed. Only those who had pulmonary edema which tended to be resistant to treatment or recurrent would be put on the steroids and then this would be properly supervised and tapered off so that you wouldn't be likely to have adverse effects to that therapy. Within a matter of a couple of weeks we put that information into a Technical Order which was then distributed at an Air Force/industry conference in Riverside, and that information then was used to build into the tech data that accompanied the Titan II weapon system in helping to guide proper use of that system. It was quite apparent that much could be done by getting good toxicological and clinical opinions early in order to meet specific needs that the military and Air Force had. About that time, attention was being directed to emergency exposure limits where you have individuals exposed to brief, unexpected, short term but measurable concentrations of a toxic material. If you can be sure that it is going to be under a certain level, then those individuals in a space or a military type duty might not have to incur the handicap of putting on protective equipment, in that they would know that they would recover from that irritating sort of environment without medical attention at the termination of that time period. But there was a condition, that before re-exposing themselves, they would have to be examined by a competent, occupational medical specialist so that there would be the opportunity to prevent their re-exposure if they had not, in fact, recovered from the initial injury. Now that type of thing was a means of using the best existing knowledge, experience and skill of scientific and technical types in solving pressing problems that were arising from a very dynamic Air Force and Space effort at that time. But it became quite apparent that many of the problems occurring medically were not occupational medical in nature. The ones that I have just mentioned were more emergency type exposures, such as your firefighters or other emergency type personnel would encounter. In fact, in the development of new systems the new chemicals and the new procedures that were introduced were frequently done by private industry and by the time the military got the weapon system those things had been pretty

well contained and the relative safety of this system had to be demonstrated or the military wouldn't accept the end product. So this brought to our attention that there might very well be a need to have better information on what was happening in the research and development in the private industrial contractor facilities as a means of understanding what to expect in the event of exposures and toxicity as opposed to studying individuals that were, in essence, in controlled industrial environments where you would have great difficulty in setting up cohorts for epidemiologic studies i.e., in having exposed groups in medium and high level versus light or no exposure. When I left the Air Force to go into academic and research work in Cincinnati in 1961 it was apparent to me that the major toxicity problems were probably going to be in private industry and not in military type exposures. Over a period of the next 10 years, first at Cincinnati and then in Pittsburgh, I had an opportunity to work very intimately with those who were responsible for programs of medical intelligence (as Dr. Jim Sterner of Eastman Kodak called their computerized medical data base). Many other companies were getting into computerization of data on their employed populations which would allow them to recognize the occurrence of adverse effects of the environment at the earliest possible time. In many instances there was a need to have a broader population base in which to recognize abnormalities and while there was willingness on the part of some companies to cooperate, there was on the part of other companies, a lack of willingness to share such data. At about the time Mellon Institute merged with Carnegie Institute to form Carnegie-Mellon University, OSHA came along. It had become increasingly apparent to us there in Pittsburgh that we were not going to be able to develop the systematic comprehensive, occupational health data system that would support American industry in the way that would be required. In fact, because of the resources being put into this area by the government it was apparent to all that if this were going to be done government would have to provide the leadership and the support in order to help insure its success. Today we are confronted with the types of problems that Dr. Culver has mentioned as being in today's paper (with regard to Herbicide Orange) and in presenting the Hatch lecture a month ago in Pittsburgh, Professor Phil Enterline put on the screen a table of random values that he had gotten from a Rand publication. He showed on one line four 7's and the line just above that three 7's and commented: "Now that is a random cluster. If that occurs in one of your plants you are going to be hard pressed to explain that that's a coincidence unless you have a means of looking at your experience and saying you have looked for other such clusters and have ruled out that possibility." Dr. Gil Collings in giving the Gehrman lecture at the Joint Academy meeting in New Orleans a couple of weeks ago commented that we have now reached the point where management and labor are both agreed that illness costs and health care costs are going to have to be solved. They are 10 times as great as the compensable illness cost and industry has

been successful in reducing the costs of compensable illness but nothing has been done to help contain the costs of the vast pool of chronic illness that resides in our population, particularly our aging adult population, the majority of whom work. So this gets at the type of problem that Dr. Wolfe was mentioning earlier whereby you have a question that's raised that involves millions of people and exposures in the past that are very difficult to quantitate retrospectively. It may cost you millions and millions of dollars to try to answer rather simple questions. Was there an adverse health effect of using Herbicide Orange? I don't know what the total cost in millions of dollars is going to be to try to answer that question and in the meantime we have gotten into sort of a crisis of public confidence so that when the facts are finally there that satisfy our scientific community, the public may have lost interest. Marshall McLuhan says that, in the presence of a crisis carried by radio and television, anyone who tries to say "I'll wait until all the facts are in before I give you my opinion" would be simply overwhelmed by the flow of events. So in many respects we can't wait. We know that is going to be much too costly and we have to go ahead and develop a system that will enable us in good conscience to answer the question "how do you know that your workers are adequately protected from the potential hazards that they encounter day by day in their workplaces"? In approaching this, much of the hard part and the costly part is in your environmental survey and control and monitoring. The medical part may very well be a very major cost if you are in fact, looking for and finding disease and titrating ability to continue work, based on whether some altered physiological parameter has returned below a biological threshold of some sort. That type of thing is done in some parts of the world. In Europe there is a much more highly developed biological monitoring system than we have in this country. On the other hand in the U. S. we have an extensive program of bio-environmental engineering (as we in the Air Force call Industrial Hygiene). To show you the extent of this when I first finished my residency training at Kettering and went to the headquarters of the material command to work under General Otis B. Schreuder and his deputy, Col. John Boysen, there were approximately a half dozen people in one office who were providing advice and guidance Air Force-wide on occupational health. One of those individuals, who is known to some of you, Col. Al Meyer, now retired, went to Strategic Air Command (SAC) headquarters. He was the only industrial hygienist at SAC. By the time he left there he had created spaces for 65 industrial hygienists, one at each SAC installation and had set up a program under Col. Herb Bell at the School of Aerospace Medicine to train engineers to fill those positions. We now have hundreds of bioenvironmental engineers, all trained and qualified in industrial hygiene and environmental survey and control supported by technicians. These individuals are collecting and storing data in a standard way under the type of program that Maj. Worthy has described to you this morning. With a little bit of extra effort in the medical area we intend to have

the COPE program, the comprehensive occupational health program add the industrial population's health status and any biological measures of exposure and lack of exposure. We will in the future be able, with a relatively inexpensive computer-run, to answer questions about clustering with regard to specific occupations and environments.

You are all familiar with the Hatch curve. I have superimposed on that the concept of threshold limit value (TLV), time weighted average (TWA), and the short term exposure limit (STEL) and ceiling value (CV). Were you not sure of your controls' adequately protecting the health of exposed individuals you might have to do more frequent evaluations. In the presence of above CV or above STEL or even above action level (usually the TLV or TWA), you usually have to perform biological monitoring tests to be sure you are not encountering occupational disease or altered physiology which may or may not be reversible. But not when you are working at levels which show that you have contained and controlled the hazard. I use as an example here the blending operation for tetraethyl lead at refineries where the equipment is so tightly fitted that 40 years of experience and monitoring the health and urine lead concentrations of the workers engaged in that operation has documented that these individuals do not have measurable absorption or excretion of lead and therefore are not at risk of getting occupational lead toxicity. So if you can come up with something that would be equivalent to the urine lead in the tetraethyl lead blenders at petroleum refineries then you may by a single measure of biological type be able to document the adequacy of the industrial hygiene control of that operation. So you would be working really in a level in which you would not expect any disease at all and it would only be where you had high values or positive findings that you would then raise the question as to whether you might not need to begin a different type of survey program. In such a case you would need to have a very carefully planned epidemiological protocol to investigate a new type of problem. Now as the comprehensive occupational health program (that Maj. Worthy has described) develops it has become necessary to develop improved guidance that is going to help doctors, especially flight surgeons, environmental health officers, and industrial hygienists, Air Force-wide, decide what types of biological monitoring to perform on our populations. When those decisions are made locally, frequently the industrial hygienist goes to an internist (or to someone with even less training in clinical toxicology than an internist) and says "what do we do?" It may be that a medical research type approach will result where there aren't any environmental data to suggest a potential hazard. To help standardize and improve decision making in all military industrial situations a Department of Defense Occupational Health Surveillance Manual was published in 1982. This manual states that where you have good environmental survey data to document that you are controlling a hazard below the action level, there is not a requirement for routine biological

monitoring. But of course a physician responsible for a worker or work force can at any time use his professional judgement to require whatever testing he feels may be indicated, but not on a routine basis. General methods of prevention in industrial hygiene are shown in Table 1 which shows the place of early detection of disease or excessive exposure. Early detection and/or exclusion of excessive exposure within a comprehensive program is usually much more cost effective than looking for early disease in an uncontrolled or inadequately controlled industrial environment. Table 2 lists some of the examples of screening approaches that have been used. Our diagnostic methods have advanced rapidly and there are much more sophisticated types of x-ray examinations and even CAT scanning that may be applicable in identifying whether chest disease has resulted from asbestos exposure. All of these diagnostic aids have costs associated with them. A simple test such as total urinary phenol in the presence of possible benzene hazard we can do internally at our analytical laboratory. But if we want to farm it out, it would cost us about \$35.00 per test. And if we were to require hundreds of individuals to have that test at a hundred different Air Force bases, you can easily see that it gets up into the hundreds of thousands of dollars quickly. Perhaps without having any benefit. So that's why we need to use care in deciding what it is that we are going to do routinely Air Force wide (Table 3).

TABLE 1. METHODS OF PREVENTION IN INDUSTRIAL HYGIENE

- . Engineering Controls
- . Substitution
- . Personal Protection Equipment
- . Education
- . Early Detection of Disease or Excessive Exposure

TABLE 2. EXAMPLES OF SCREENING APPROACHES

- . History
- . Physical Exam
- . X-Rays
- . Pulmonary Function Studies
- . Audiograms
- . Laboratory Tests - Blood, Urine, Feces, Hair, Expired Breath, Etc.

TABLE 3. CRITERIA FOR EFFECTIVE SCREENING

- . Screening Must Be Selective
- . Must Identify Disease In Its Latent or Preclinical Stage
- . Adequate Followup
- . Good Reliability and Validity
- . Benefits Should Outweigh the Costs

Obviously in deciding on a biological screening test we must be selective. It must identify disease in latent or pre-clinical stage if we are looking for disease. There must be provision for adequate follow up. There must be reliability and validity and the benefit should outweigh the cost (Table 4).

TABLE 4. SCREENING TESTS USED TO DETECT BIOEFFECTS OF LEAD

- . Hemoglobin/Hematocrit
- . Stippled Red Blood Cells
- . Coproporphyrins In Urine
- . Free Erythrocyte Protoporphyrins (With ZPP)
- . Porphobilinogen In Urine
- . Delta - Aminolevulinic Acid (ALA) In Urine
- . Activity Of ALA - Dehydratase In Erythrocytes

In the case of lead some of the parameters that are commonly used are listed. Some positive results suggestive of alterations in physiology (in erythropoiesis, particularly) can occur at very low levels of exposure, and they are not specific indicators of lead effect, per se. The blood lead and urine lead usually give better information for what we need to know than do such measures of altered physiology (Table 5).

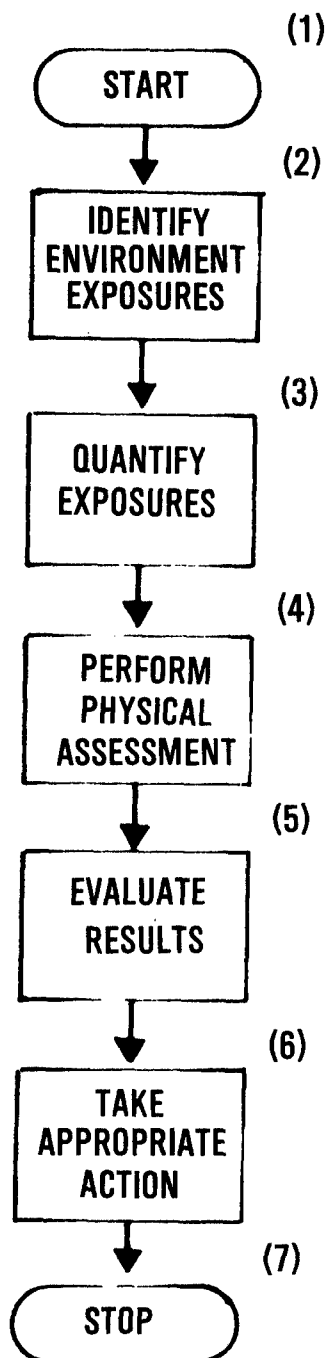
TABLE 5. TESTS USED TO MONITOR LEAD EXPOSURE/ABSORPTION AND BODY BURDEN

- . Lead In Whole Blood
- . Lead In Urine
- . Lead In Urine (After Chelate Challenge)
- . Lead In Hair

Figure 1. Generic occupational health algorithm.

In the case where there is a question whether someone has had exposure and increased body burden as a result of lead exposure and it is necessary to make a decision, the use of the chelate challenge and subsequent measure of lead in the urine has been recommended and may have a place. Lead in hair is a possibility. Some of these biological techniques may be very useful in solving individual problem cases but not be appropriate for routine use in an occupational health surveillance program. Lead in the whole blood is the most valuable single indication of inorganic lead absorption. In the case of organic lead absorption, however, even in the presence of acute clinical disease the blood lead will usually be normal. It would be only through increased urine lead that you would have an indication of overexposure to an organic lead compound (Figure 1).

One of my associates, a reserve AF medical officer, Col. Al Harter whom some of you may know, is the Medical Director of GE-Erie. He was formerly with NASA. When he was on active duty with us during the past 2 years he used his knowledge of computer programming to help systematize some of the types of guidance that we were developing. He has worked with Don Worthy in this. It's a collaborative effort. The end result should feed right into the COPE program. It is not completely worked out and is still developmental. But this summary will give you an idea of what we are doing. And from knowledge of our needs you should have a better understanding of some of the demands that we will be putting on the scientific field, particularly in toxicology (Figure 2).



Now this generic occupational health algorithm has the type of guidance in the configuration of the boxes here that is meaningful to programmers. The branch tree decision-making is intended to put it all together in the initial placement: into a relatively controlled occupation (A) or in a population that may require protective equipment (B) or in a population that may have unpredictable exposures that it is not possible to protect against (C). The followup medically in these cases is different. Ideally, what you are trying to do is get them back into group A which has the least requirement for medical surveillance. But in the event that they are group C or B, you may have a requirement for close follow-up including performance of diagnostic workup until the individual is either disposed of by permanent removal or return to full duty or something in between. Now obviously this is a crowded figure. (Dr.) Col. Stoller, who is the occupational medical chief at the Air Force Logistics Command at Wright Patterson, has published an article intending to guide physicians of all types in the logic of including evaluation of environment along with biological findings in reaching decisions on occupational health surveillance and placement, periodic exams and disease evaluation. His is considerably more complex than this, and even this is too "busy". So (Figure 3) what we have done is to reduce the complexity by taking from the generic and then going into details that are specific for the material. Then at an appropriate point we go back to the generic for detailed guidance. This would of course be accompanied by notes and written guidance. This example using benzene is included here mainly to help show how the data obtained would be evaluated, used and stored (Figure 4). In the case of mercury what we are showing you here is a type of professional decision that has been reached by our staff as a result of reviewing literature in light of our own experience. What we have come up with is meaningful to us. It may not be meaningful and acceptable to others and I can see a need for the same sort of consensus review in this area as has been applied to the environmental thresholds that have been prepared, TLV's particularly.

We can help to structure and to further progress in this area by doing some of the foot work. Then it's mainly going to be necessary for others in private industry and in other branches of the military service and in the academic and scientific communities to cooperate by looking over our shoulder. If there is a better way of doing what we propose to do either from the standpoint of accuracy, reliability or cost effectiveness then we certainly need to communicate on that before we apply it Air Force-wide or military/DOD-wide (Figure 5).

This is the inorganic lead and next for organic lead. I don't expect you to read all of this in the short time that we have. You can follow the decision tree here. If you have over 40 mg per decaliter in the blood you would repeat it at more frequent intervals. Then depending on the outcome you would get into the appropriate type of detailed followup until you could

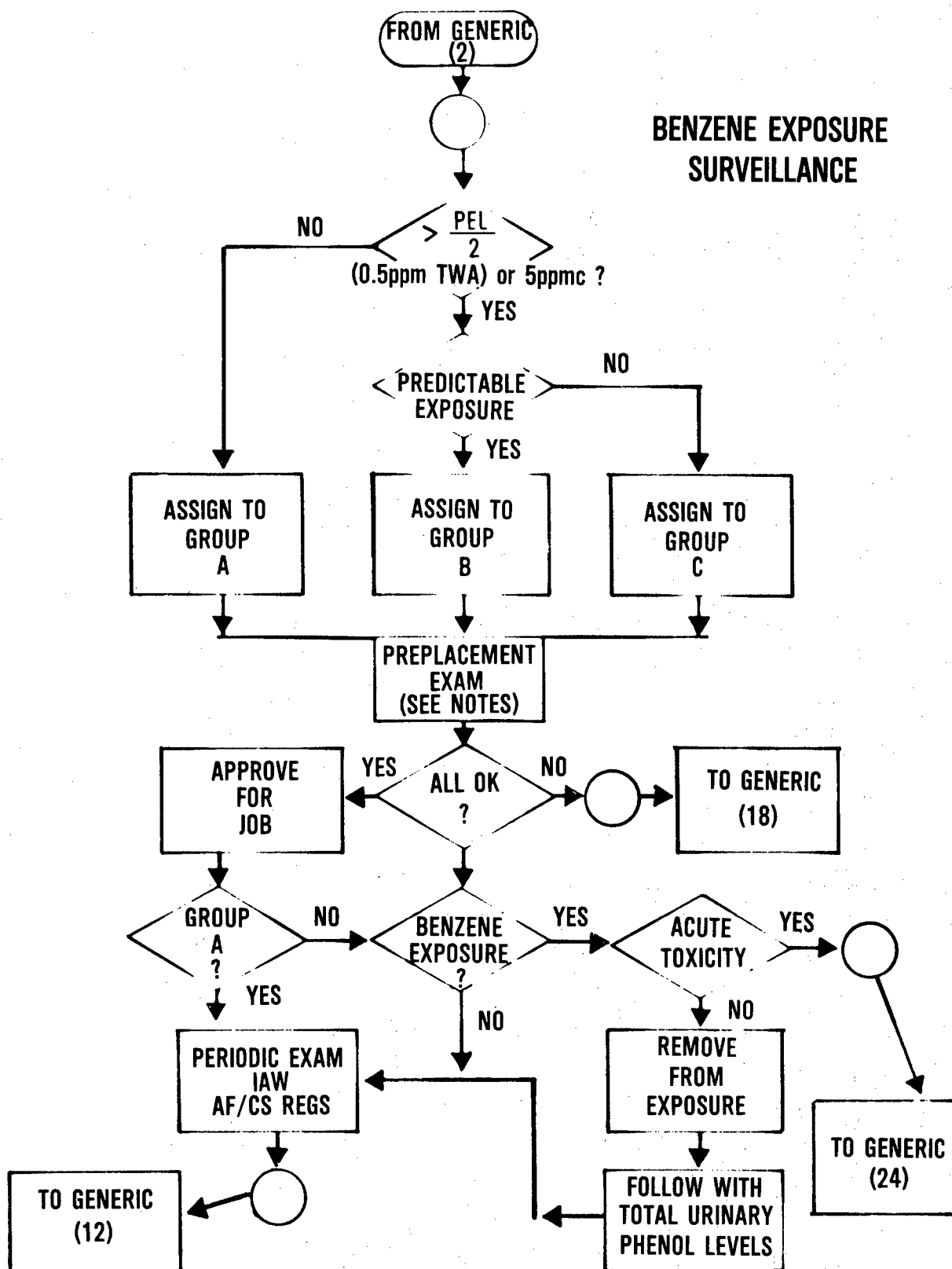


Figure 3. Benzene exposure surveillance.

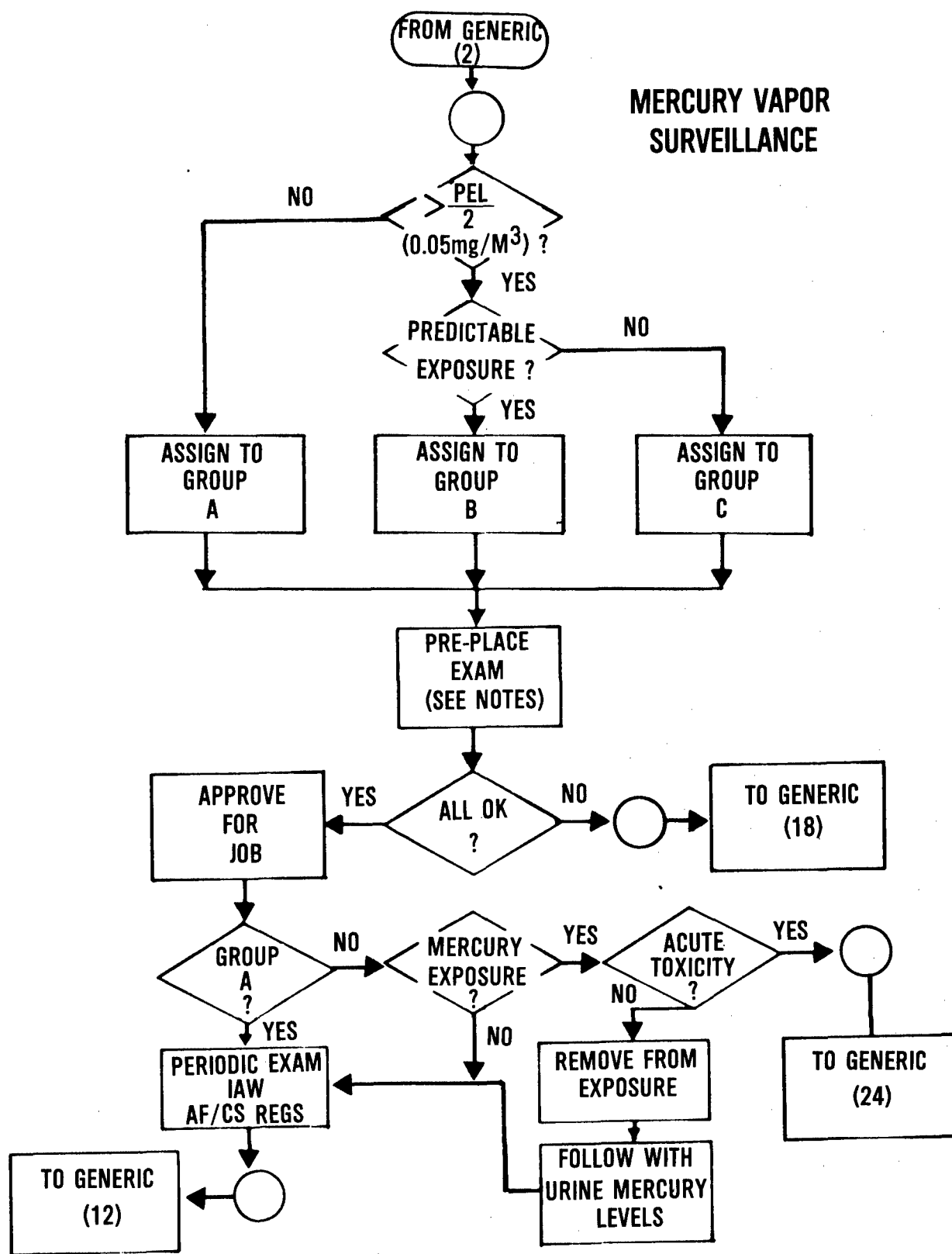


Figure 4. Mercury vapor surveillance.

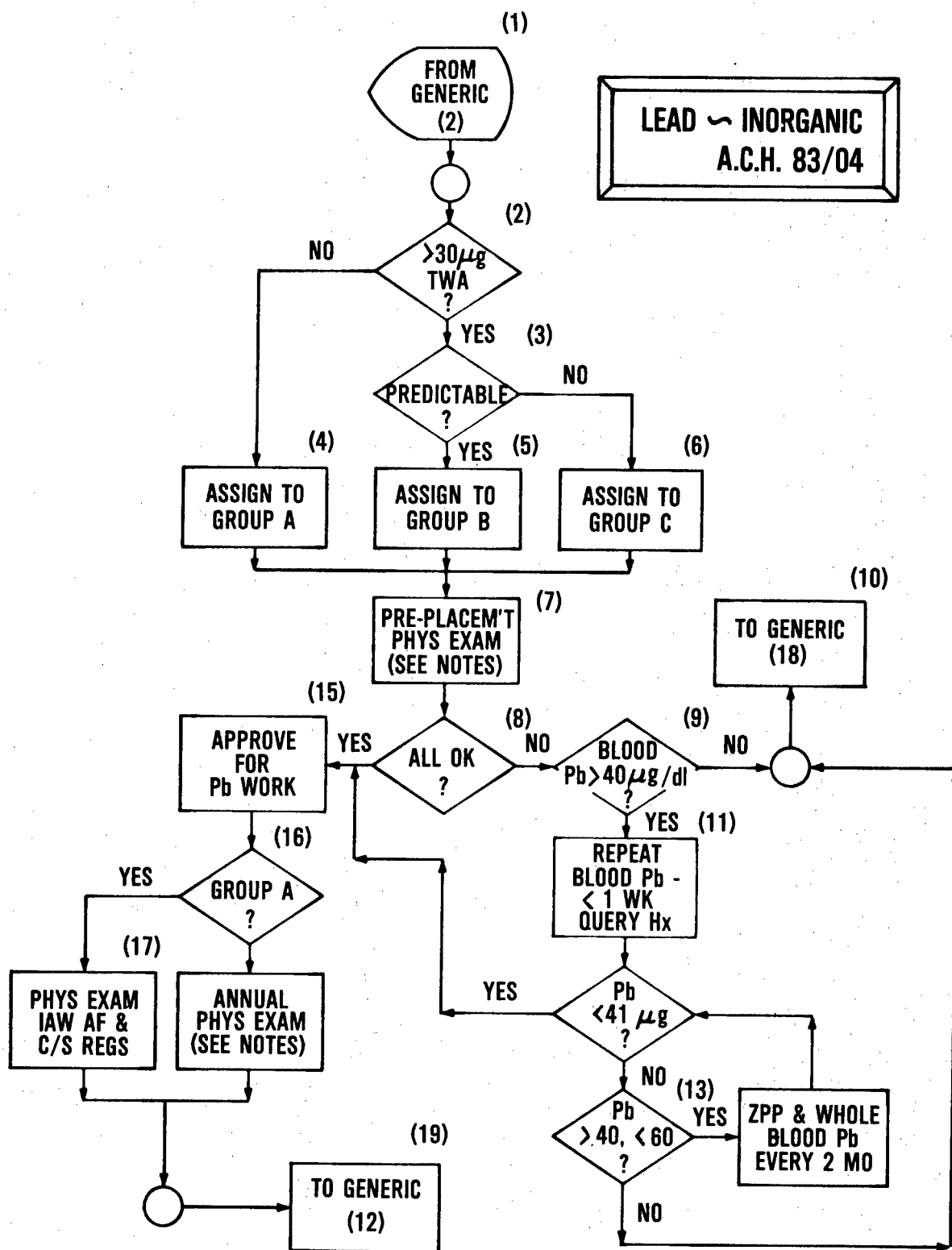


Figure 5. Lead ~ Inorganic A.C.H. 83/04.

eventually document that the condition had been brought under control and that the individual could be returned to the regular, routine type of monitoring. This has the advantage of allowing an audit, which could be very valuable, while allowing evaluation of data, looking for clustering. At a later time, it may also help support statistical and epidemiological research (Figure 6).

Here we use the urine because the blood values are not useful (in organic lead) (Figure 7).

In the cases of chronic acid and asbestos (Figure 8), you can see how much simpler and easier to follow this is when you can reduce it to a smaller number of items in the algorithm relying on the generic for the more complicated follow up (Figure 9).

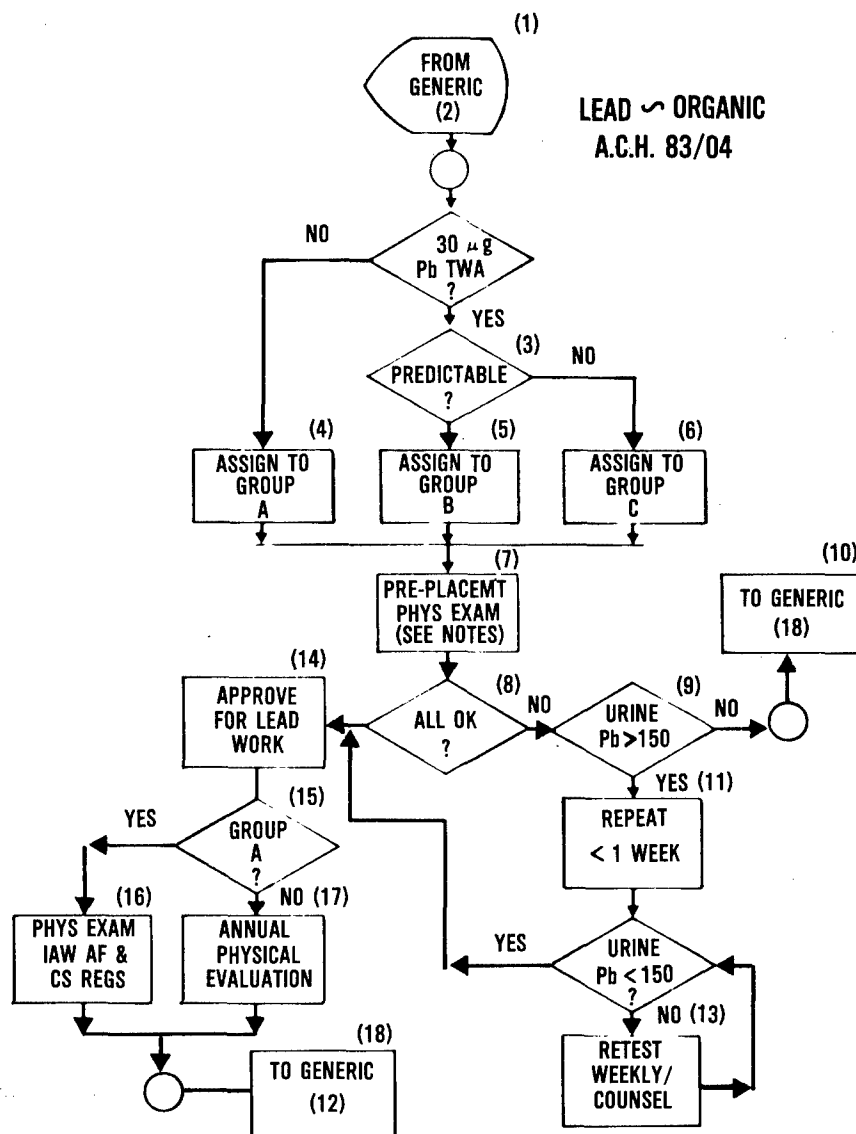


Figure 6. Lead - Organic A.C.H. 83/04.

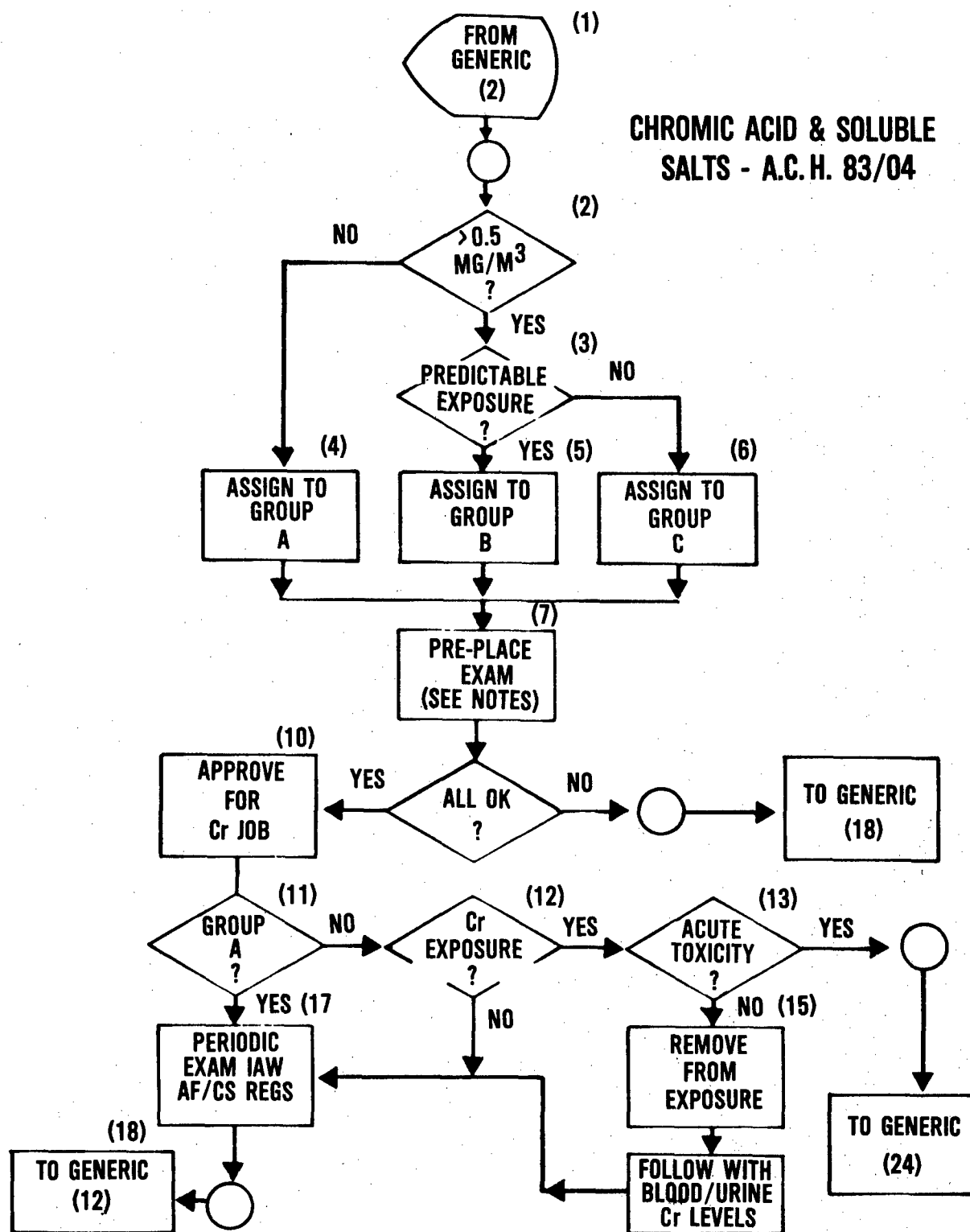


Figure 7. Chromic acid and soluble salts - A.C.H. 83/04.

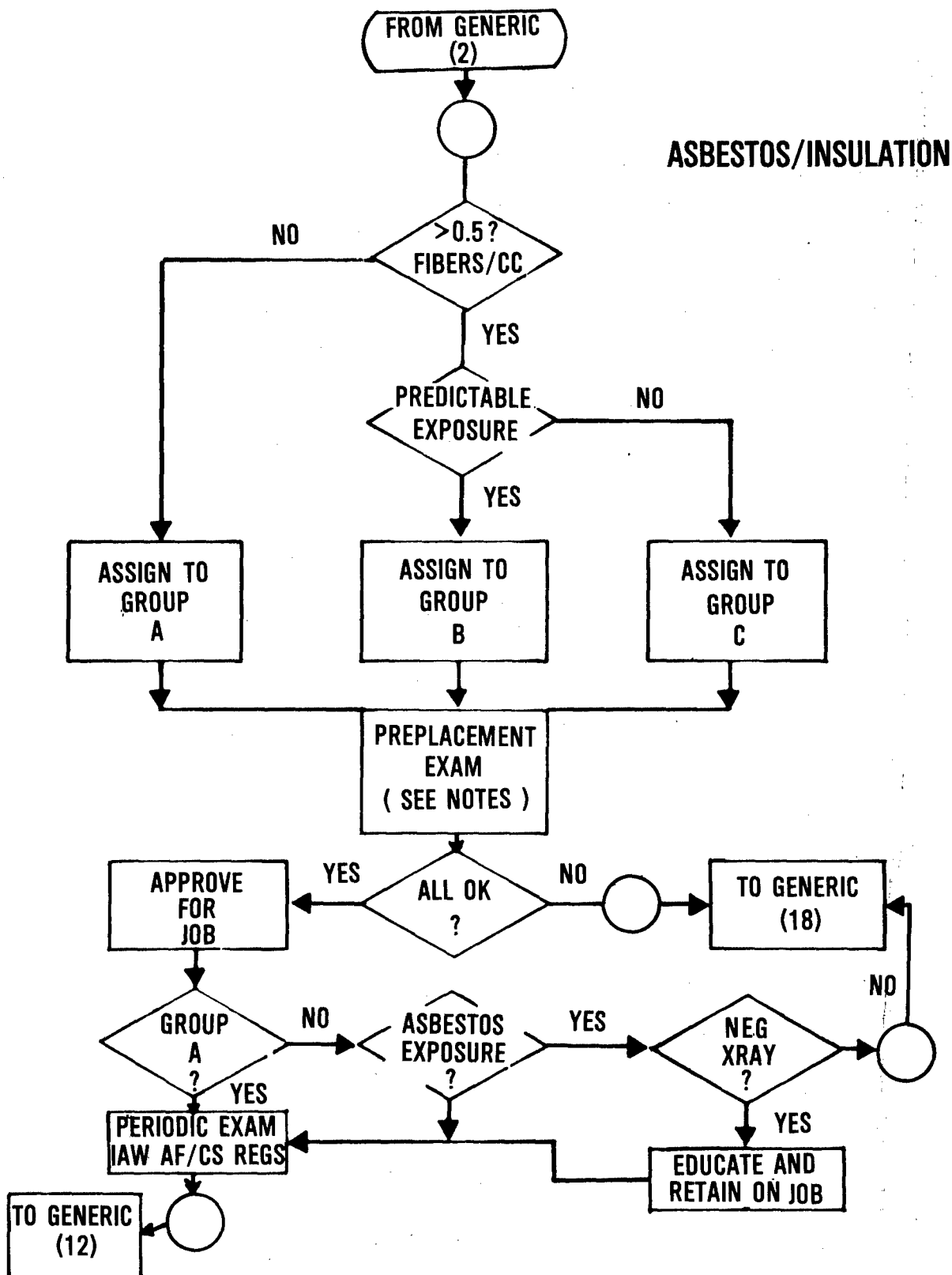


Figure 8. Asbestos/Insulation.

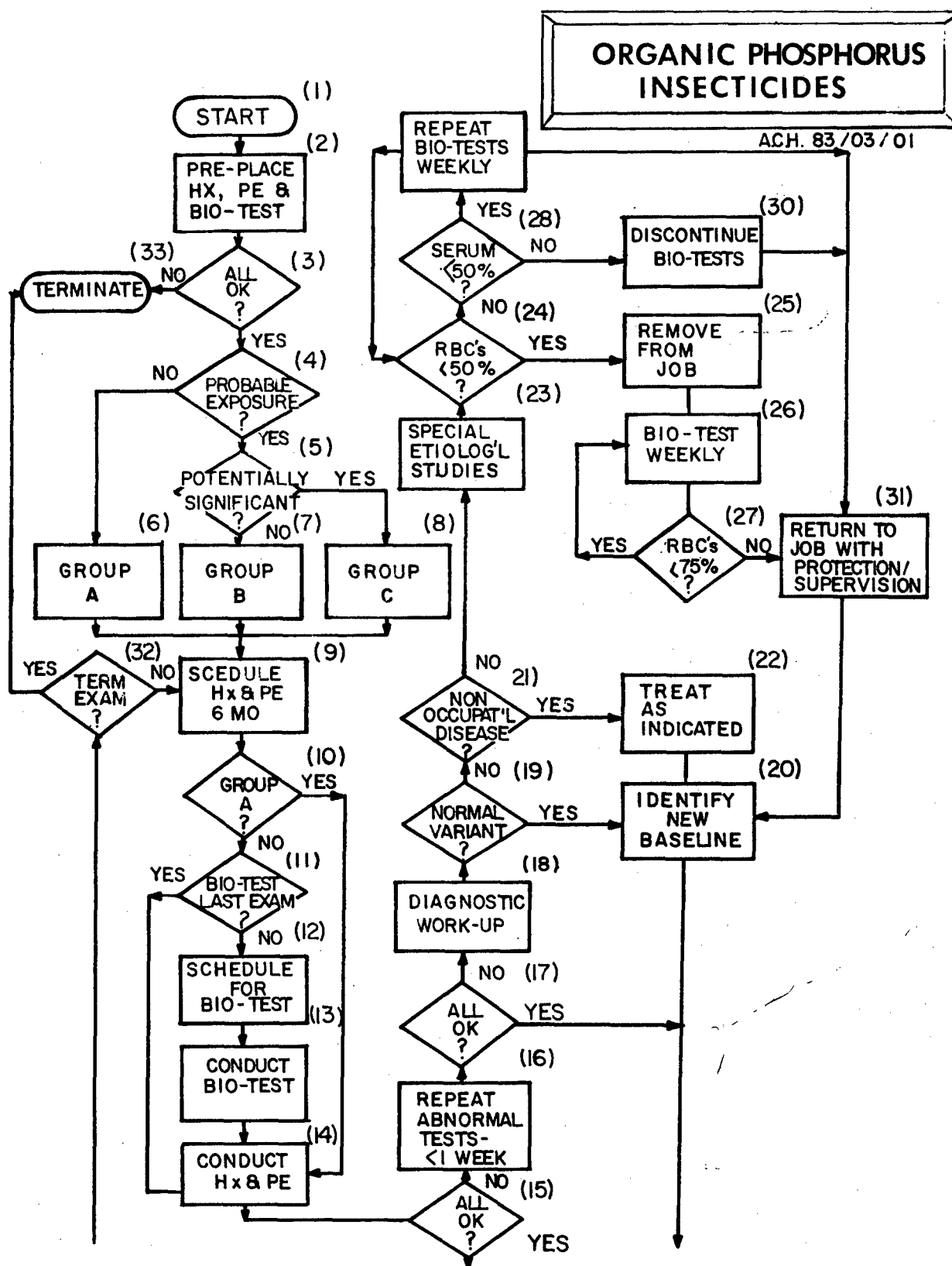


Figure 9. Organic phosphorus insecticides.

I put this one on last because Al Harter developed this one (on organic phosphates) just before the generic. I think that it was so "busy" that he decided there must be a better way of handling this and so he went to the simplified version with the ability to separate out modular sections for more careful study on the routine occupational health surveillance. I think you can see that this crowded format does not provide good guidance because it would take even the initiated awhile to go through it. The uninitiated would find it a jungle.

I would just like to say in conclusion that we should be in a better position to document the effectiveness of our health conservation efforts. Not just hazard control but health conservation efforts in general. Guiding future efforts in solving a whole new series of problems will undoubtedly involve more immunology and geriatrics in supporting aging populations. We need to be prepared to deal with these. Professor Ted Hatch, when he retired from the University of Pittsburgh's Graduate School of Public Health said: "If I come back in ten years and you are still teaching the same thing I'm going to be disappointed in you." I think each of us must take that to heart because we have a dynamic scene and moving targets.

In closing, I would like to recognize with thanks all assistance so far received from professional and scientific colleagues from within and without the military services many of whom have gone to their final rest, having passed the gauntlet to us. In particular, I should like to single out my associate in Occupational Medicine, Maj. Dana Mirkin USAFMC who helped prepare the figures and was prepared to present the talk today on this subject if it had proved impossible for me to do so. He, too, is an alumnus of the University of Cincinnati's Kettering Laboratory in the Department of Environmental Health and is sure to be an important contributor to future progress in this area of mutual interest and concern on which Dr. Culver has asked us to focus today. Thank You.

OPEN FORUM V

Dr. Crocker (University of California, Irvine): I'd like to ask the first speaker, Maj. Worthy, about the inclusion in your overall data base of a toxicology component which I thought was an important and interesting thing to take into account. At what stage of development is that in? I am sure, for example, that members of this audience would be willing and happy participants in the development of such a data base, though I'm sure you also have sources like the material safety data sheets and other such sources. Could you describe how much additional toxicological input there is and if you have any plan to seek it from a source such as the Toxic Hazards Division?

Maj. Worthy (USAF OEHL/EC): Yes, sir. We realize that our data bank has at least two uses. We must use this data or the information of something we call either applied toxicology or operational toxicology in our industrial hygiene program. We also realize that if we want to improve this information in the future we must be in a position to provide data to use in future toxicology studies. Basically, our data or information comes from sources that are used in the Manual program. The Department of Defense takes information from sources such as the NIOSH publications and consolidates it and puts out the DOD Medical Surveillance Manual. We use NIOSH'S RTECS to build our own data base. In the future we are going to have to take data from many sources and build a toxicological data base. The Navy is working on a very comprehensive program out in San Diego right now where they are compiling the questions you would ask in a medical exam, the chronic effects, the acute effects and various other aspects for a complete profile that are going to be extremely valuable. We hope to utilize that program at the proper time. There is quite a bit of work going on in the other military services.

Dr. Thomas (SES, Retired): I would like to ask you one question, Major. Did your group ever consider placing a terminal in the Toxic Hazards Division to receive direct input and to interact, because they need field exposure data in recommending various kinds of limits to the Surgeon General. The only way we find out how good those recommendations are is if we can later see the field exposure data, if you have some handle on the exposure duration and frequency and perhaps see what the difference is in the biological response between the rat, the dog, the monkey and finally the human. It would be an immense help if there could be a terminal here so that the bench scientist who works on a certain Air Force compound could get the exposure data. I tried many times to obtain factual information about exposures but it is awfully hard to obtain. Of course the system isn't working yet, but it's so easy to interact with a computer that I recommend making that connection available to the researcher.

Maj. Worthy: Yes, Dr. Thomas, there is definitely a plan to accomplish that. From the hardware standpoint we are planning on using computers at each of the bases and these would feed into a central host computer data base. In other words, the Base terminals would feed into a management program in the central host computer where we would store the data base and both the School of Aerospace Medicine and the AMRL will have access through terminals to this system.

Dr. Mukerjee (U. S. Environmental Protection Agency): This regards Dr. Wolfe's discussion on Agent Orange. First, regarding the epidemiology of cancer in this country, the American Cancer Society in 1982 revised their estimate of how many Americans eventually will have cancer from 25 to 30%. This is a different subject than Agent Orange. When you mentioned the study on incidence of cancer amongst the Swedish population by Lennart Hardell, I was surprised that you didn't mention the estimate which was done this year in this country by NIOSH in an occupational exposure. Their studies of incidence of soft tissue sarcoma tallies very well with that of Hardell's study of a Swedish population. Furthermore, regarding the reproductive effect, there is no doubt that the Canadians and Australians couldn't find any high incidence of reproductive effects in their Vietnam veterans. However, Dr. Haniffee published a paper in 1981 in Science in which he reported that there were people who were exposed occupationally or from the environment in New Zealand who had a higher incidence of reproductive effects. I would like to find out how you would determine the exposure precisely because if you don't know the exposure it will be very difficult to do the risk assessment on your population. I would like to know that because it is not only the liver or kidney which accumulates 2,3,7,8-TCDF but also the adipose tissue accumulates considerable amounts.

Lt. Col. Wolfe (USAF SAM/EKE): In regard to the cancer epidemic, cancer is, of course, the number 2 cause of death. However, if we could delete the cancer problems caused by or associated with cigarette smoking I think, and many other folks feel, that the cancer problem would largely revert to normal. Not normal, but would largely disappear because death due to many of those cancers other than lung and smoking related cancers are dropping year to year. The soft tissue sarcoma is another very difficult area. For every study that shows possible problems from Agent Orange and the phenoxy-herbicides and dioxin, there are other studies that show a lack of problems or negative response. Most of these studies in the past have had major weaknesses and were done when sophisticated epidemiological and statistical approaches were not available and the definitive studies probably never will be done. We're dealing with human populations and it's always difficult to do the perfect study, unlike animal studies where you know you are dealing with observations rather than experimental design problems. I think that what has to happen is that we look at the total picture, combine the effects of many studies looking for reduplication of results,

consistency of results, consistency of study designs looking to find the real answer. It is a problem. The NIOSH study used combined populations. They took a study of Dow workers and they took a study that Monsanto did and pooled the groups and looked at the incidence of soft tissue sarcoma. Statistically that has many drawbacks as well. We're dealing with major problems there. The birth defect issue is very difficult to sort out because it is so emotionally laden. In our study we have interviewed 2300 subjects for three hours in their homes. These were done under contract with Lewis Harris from New York. They went out and interviewed their wives for an hour each and then went back and interviewed former wives and other women that had been involved in a significant manner with these gentlemen. We have collected a very extensive reproductive history and we are in the process now of looking at that data trying to sort it out and sort who was the father and who was the mother of which child in order to look into the effects of smoking during pregnancy, and alcohol use during pregnancy and the other multiple confounding factors in birth defects and infertility and fertility. Our exposure estimate, we feel, is somewhat unique from all the other studies of Agent Orange. One of the problems that CDC is going to face is that they only know roughly where the herbicides were sprayed. Our pilots went out and sprayed the herbicides where they thought they were supposed to. We know where company headquarters were located. We know groups of 100 people that were in the field and we know where those locations were in relation to the spray in time and geography. Unfortunately, the operational units were on the order of 10 or 15 or 20 people at a time and they would fan out for hours or for a day or two at a time from their headquarters location. When CDC says here's a group of 100 people that were at this geographical map coordinate, they may not really have been there. In our study the people stayed at one place. They stayed at a base and did a specific job. We know how much herbicide was sprayed every given month from 1962 through 1971 when we began using arsenicals exclusively. We know by month what was sprayed, how much of it was sprayed and we know how many people were there doing that individual's job. If his job was to repair aircraft we know that during this month there were 10 other people doing that job. We know how much was sprayed that month and making the assumption that everyone did his fair share of the work, we can come up with sort of a crude estimate of the number of gallons that that individual potentially contacted, and if, in fact, the other people doing his same kind of work had the same potential, we can develop an exposure index. It's not ideal industrial hygiene data because we don't have air sampling measurements. We don't have skin swatches and patches, but within some limits we feel we have a very good estimate of the potential exposure of these individuals and this is what we're using in our study.

Dr. Culver (University of California, Irvine): Dr. de Treville has a comment with regard to this also.

Dr. de Treville (USAF, OEHL/QE): One matter that is of real concern to many of us is the discrepancies between some of the studies that have been published both in toxicology and epidemiology. Dr. Bert Dinman, who is head of the Alcoa medical program, has proposed a system called an ionogram for exercising quality control over what has been published and what has been done and awarding a value to each of these studies and then adding them up and then, depending on the numerical outcome of this calculation, saying whether this was a carcinogenic material or not. Dr. Dinman's publication on this came out in the Journal of Occupational Medicine in either the July or August issue this year. Obviously, this matter of quality control is something that is going to be extremely important and it may be that individuals will be at a loss to be able to apply the approach that Bert has suggested. But perhaps within a group such as this, it might be possible to tackle it along the lines that Dr. Thomas was suggesting. In other words to keep in close touch with progress and where it appears that this group can do it better and faster and come up with good results to be prepared to step in and help further that progress and I think in many respects this is needed. I would like to present as a challenge to this group that it review the Dinman approach. You may want to modify it. In fact he said that this was a somewhat simplistic approach but at least it was an approach. It's going to require great skill and ability to use quality control and to communicate the results and I would think that that would be appropriate for a group such as this to consider.

Dr. Mukerjee: I do agree with you that the quality control of the data is very essential. The quality of work which has been done in Sweden was extremely well controlled. The pathologist who observed the slides is well renowned and Hardell is a well recognized epidemiologist. So there is no qualm with the observation which was done in Sweden but the bottom line should be that scientific data should be validated and there should be quality control.

Dr. Klemme (NIOSH): This question is directed at Dr. Wolfe. A year ago at this conference, someone, I don't remember who, presented animal data following exposure to either TCDD or dibenzofurans. The report was of delayed acute type response of essentially a failure to thrive but with no clearly defined pathological lesion. But ultimately animal death at some period later than would have been expected from an acute toxic response but otherwise fitting those definitions. Someone in the audience, a physician from the Army Environmental Hygiene Agency, commented at that time that some of these reported signs and symptoms of the animals seemed to correspond with some of the vague reports of findings in humans exposed or reporting exposures to the same chemical. These were things that a physician generally would not pay a lot of attention to because they are hard to define but they are the sorts of things that in a general clinical sense rarely have importance. In your presentation you

used a vernacular term "the dwindles" to describe a symptom. Is this something that you have seen or is being seen and how are you addressing it?

Lt. Col. Wolfe: The "dwindles", this esthetic approach, is recognized fairly commonly with acute and subacute exposures. It's been seen in the industrial plants of Monsanto and Dow after some of their industrial accidents. But on a long term basis it is very difficult to measure. We're approaching that matter in our study with psychological testing, looking at some of the subtle approaches toward pre-clinical measures of abnormality in some of the laboratory procedures. This includes liver function tests and many of the others that we're doing. It is difficult to approach. Again it's the sort of thing that is somewhat age dependent. Psychological overlay has major effects on that. Depression, anxiety. The more you hear about issues such as Agent Orange the more people who think they were exposed tend to be concerned and the more anxious they are, the more they read, the more anxious they get. The more anxious they get the more they read and it is very difficult to sort it all out.

Dr. Klemme: Let me just ask a follow-up question to that and maybe direct it to others here who would be familiar with the toxicology issues. Insofar as the laboratory animals were not experiencing a psychological overlay, is there further information to be had about what the effects are in a controlled toxicological setting? Is there something more definable or approachable by biochemistry rather than behavioral studies?

Lt. Col. Wolfe: That's a tough question to answer. As you say, the animals are surely not reading the papers and watching the evening news. But people are and I think there may be problems here. We're looking 10 to 20 years later for chronic effects. Acute effects are one thing with these sorts of chemicals but chronic effects are another issue. There may be carryover and then again there may not be and that's one of the things we are trying to determine.

Dr. Yang (NEIHS, NTP): I have a question for Dr. Wolfe. It seems to me the ultimate challenge for epidemiology of Agent Orange would be to study the Vietnamese population in South Vietnam. So far there doesn't seem to be any activity in that area. Am I right or wrong?

Lt. Col. Wolfe: That's true. There was some research done there. Dr. Tong from Hanoi was doing some work mainly looking at clinical descriptions. He looked at the reported incidence of deaths in hospitals due to primary liver cancer. Prior to 1965 and after 1965 he looked at birth defect rates but by and large reported observations without epidemiological adjustments and approaches, without looking at confounding factors such as aflatoxin exposure and other sorts of family backgrounds of other birth defects continuing within a family. He was in the process

of trying to gather some international support to fund more in-depth studies but unfortunately died of a heart attack and that work has not been picked up by anyone else yet. One of the problems is identification or clearly identifying who was exposed and who was not. A lot of Vietnamese were working with the operations loading the aircraft, handling the bulk herbicide but those records, if they existed, were lost and those folks, if they are still available, in the current political climate may be unwilling to volunteer that they worked closely with the American forces. It's a very difficult situation to work in over there. It would be an ideal group to study if the identification and quantitation of exposure could be worked out.

Richard Henderson, Ph.D. (Consultant): First, I'd like to make about the same comment I did two years ago and that is that in looking at the health of populations, one of the major factors is nutrition. We now have computerization that will allow us to take a food intake record and look at the percent of essential nutrients that people are getting and I think that we need to do a good deal more of this type of looking to see what effect it may have on disease. I would like to expand on the comment that I made two years ago at this meeting. We did a little of this on a pilot basis. I've looked at four day diaries of food and beverage intake of about 90 employees. For years, the American Heart Association has recommended that not more than 30-35% of calories come from fat. More recently the National Academy of Science report on Diet, Nutrition and Cancer said that not more than 30% of calories should come from fat. Out of 90 employees we studied we only found two that were getting less than 35% of their calories from fat and only one that was getting less than 30% of his calories from fat. One employee consumed over 60% of his total calories from fat. In looking at these records we found that without special supplementation, someone on a 1000 calories per day diet trying to lose weight is highly likely to have deficiencies in essential nutrients and in some of the B complex vitamins and the trace minerals and we have documented this. In terms of looking at any population one of the factors that should be looked at is the nutritional status. Individuals tend to follow a fairly fixed dietary pattern and unless they are acted upon to make them change their dietary habits then a few days at looking at what their eating habits are gives a pretty good measure of what their nutritional status is.

Dr. MacEwen (University of California, Irvine): I have a question for Dr. Wolfe. You described your population in your Ranch Hand study very well and you are doing an indepth study of both them and their wives and former wives or housemates. What I'd like to hear a little bit more about is your control population. I presume they are a military population or past military population. Are you doing the same kind of indepth study on the fertility of their wives, their former wives and other special associates?

Lt. Col. Wolfe: Right. Our comparison group comprises 30,000 individuals who did the same kinds of work. We matched the exposed and control population on age so that 98% of our two groups match within three months of the date of birth and 65% match exactly to the month of birth. We matched 10 control to 1 exposed so that we would have a very large pool. We matched on age, we matched on race, we matched on type of job performed. We matched pilot to pilot, navigator to navigator. We matched enlisted ground personnel to other enlisted ground flight mechanics. We matched enlisted flying people, load masters and such to other load masters. Folks that flew similar aircraft. It was not the same aircraft, one was a two engine propeller-driven cargo plane. The comparison group was a four engine propeller-driven cargo plane. We tried by matching on military career job category and we think we came fairly close on matching many of the subtle psychological factors that make a pilot different from an administrative person. We decided that fighter pilots would not make good comparisons because anyone that's dealt with the Air Force knows that fighter pilots are different to start with. They are unusual. Their psychological motivations and patterns are different from others but we did match cargo pilots to cargo pilots and everyone was handled in the same way. The interviewers and the physical examination physicians and the people examined had no idea of the status of these other individuals. The comparisons were made on the examinations and the questionnaires of the controls at the same time as the exposed group and none of the contractors making the comparison were aware as to who was who.

Dr. MacEwen: I'd like to ask one more question if I may. Besides Agent Orange, what other chemicals have you or are you planning to study in this kind of manner?

Lt. Col. Wolfe: We've got the other herbicides, of course. We can use the same material and the same exposure index to look at the arsenical herbicide. We will be able to look at the people who sprayed malathion for malaria control as well. We could look at that. I personally would like to redefine the group and look at those with evidence of hepatitis B to see what their long term health has been like. We've got a three hour interview and an extensive military background and history. We've got lots of factors that we can look at once we get over the crunch of getting the word on Agent Orange out of the way.

Dr. MacEwen: I didn't quite finish that last question. I was referring more to compounds of which the military is the primary consumer, such as UDMH or other fuels of that nature. Are there any long range plans to follow those kinds of populations to see whether there are measureable consequences from their exposures? The literature contains the possibility that some of the materials may have carcinogenic actions or other long range effects and I wonder if there are any plans for long range examination of former military staff who might have been exposed to these kinds of things?

Lt. Col. Wolfe: I'm not aware of any specific plans at this time. I don't think our Ranch Hand study group would be appropriate for that right now. As the computerized occupational health program gets underway, that possibility will open up to us. Because then we should be able to identify groups of individuals that performed a certain job or worked with certain chemicals. The hardest thing we had to face was identifying our population at risk. It took about 2 1/2 years to find out who really was there and what they did while they were in the service and the computerized program that Major Worthy described would make that far easier when this program gets underway. I think those avenues will open up to us and decisions can be made to conduct epidemiologic studies of those sorts of exposures.

Dr. Culver: I'd like to ask a question of the audience. Major Worthy's presentation showed a slide on trichloroethylene and it listed SGOT changes as a function of exposure to the trichloroethylene that were well below the normal population reference values for SGOT but showed progressive changes over a period of time. What can the toxicologists who have worked with trichloroethylene tell us about the significance of those changes as seen in animals? This I think is an example of the kind of question that is going to be coming more and more frequently to you from information systems such as are developing now.

Dr. Torkelson (Dow Chemical): I can't answer the question but I can raise two questions. One, was that an individual or was that a group that was showing a change?

Maj. Worthy: We tried to do both. A small group and an individual.

Dr. Torkelson: As I recall the change was in one individual rather than with the group.

Maj. Worthy: The adverse result was from an individual, yes.

Dr. Torkelson: It was not an epidemiological study. I guess the problem that's bothering me here is that it is going to be possible for those who are not competent to abuse these data. I can conceive of a situation where someone goes into the data bank and does an uncontrolled study and arrives at the conclusion some chemical does cause an increase in SGOT and he pulls out whatever data is in the computer without controls. Now you have created a problem, you've created an issue, I should say. Maybe he should have looked for whatever chemical was handled by this same group of people. Because if they are using one chemical it may well be that just by coincidence or not by coincidence, it may be just by design they are also getting simultaneously exposed to a second chemical, which in fact is the one that's causing the change. A competent epidemiologist will recognize this and try to rule out this sort of compounding. Is there going to be control of your data base to prevent its abuse?

Maj. Worthy: The data base, when consolidated, will be very much controlled. We envision moving the data out of the Air Force Base level because that's where we figure the most abuse would come. They are not going to have available the entire Air Force data base. They are only going to have their own data that is generated at that particular base. Plus they are only going to have say 2 to 5 years worth of data. In other words, they can look for trends but they cannot do any studies. They would only have enough data that would point them in a direction that something must be investigated but would not be able to come to firm conclusions. The only people that would be drawing conclusions from the data in the central host would be the research community.

Dr. Thomas: I understand that military personnel again are going to be moved around as usual and even maybe at a faster rate. How will you follow up on an individual propellant handler or somebody who works with degreasing through the various bases he is assigned to? Will that be easy to pull out from the records in a central computer?

Maj. Worthy: Yes, because every individual has his own identifier, his social security number and job category number and no matter where he was or where he goes he will still use the same identifiers. All of this information will be in one central data base. It will be simple to match wherever he is.

Dr. Yang: I'd like to make a clarification of a point and also expand on Dr. Torkelson's comment a little bit. First of all, I thought the slide you discussed was about trichloroethane and not trichloroethylene. Is that correct?

Maj. Worthy: Yes, that's correct.

Dr. Yang: Secondly, I want to expand on Dr. Torkelson's comment about the possibility of another chemical. As I recall, on the slide, this person smoked one pack of cigarettes a day. I don't know what the drinking habit of the person was and I don't know what the prevalence of drinking in the Air Force is per se. However, since this is quite a common habit it brings back to mind the lecture yesterday from Dr. Laham about perchloroethylene and ethanol interaction. It seems to me that that there could be quite a similar situation in Air Force personnel after exposure who go home and have some whiskey and so on. That could be a very interesting kind of a situation to look into.

Maj. Worthy: On that slide, we also showed this individual to be a moderate drinker and in fact we could not make any conclusions. If anything, the correct procedure would be to go back to this individual and do more extensive clinical tests. We could have done an entire liver profile versus just the one test. It was just strictly a one shot screening scenario to sort of see if we really had something which might indicate the need for a more detailed study.

Dr. de Treville: I was just going to comment that in one instance it was brought to my attention that the use of the SGOT liver function test was creating problems because of inability to get the individual to return to a baseline and it reminded me of Dr. Tamburo's experience with the vinyl chloride workers where there were something like 15% of the work force that just genetically could not get below the norm. To apply that test to a group of employed skilled workers and separate out 15% of your work force because they couldn't return to a normal "value" would not really be in the best interests of anybody. Dr. Tamburo argued in favor of some flexibility. Not putting these tests into the law, as was done with vinyl chloride, but allowing some flexibility for scientific and professional variability. In his work he did some excellent studies that showed that the ICG dye test was much more accurate in indicating early liver changes which were compatible with histopathologic changes revealed by needle biopsies. Where you have those types of data it's reasonable and necessary to use the best information rather than going with something less selective.

Richard Henderson, Ph.D.: What is the meaning of a value? If you get a SMAC 22 printout of biochemical tests it will give you the normal range to expect but that's the normal and it doesn't take the outliers into consideration. I don't know as we have too much knowledge of what can we expect on repeat tests from the same individual. I'm sure that some of that information is available but I am not sure that there is enough of it available to really do a good evaluation of some of these numbers at the present time. Dr. de Treville touched on this matter in terms of random numbers. What can you really expect? Are you going to see a high value occasionally? How often? What is the significance of that? I think in terms of the work we did on urinary mercury determinations. We knew that if we got a 0.3 mg per liter value it could be on the way up or on the way down. If we want to control the exposure at 0.5 as an absolute cut-off then we've got to repeat a spot sample at 0.3 mg/L. I'm not sure we've got the same type of information but I'd like some discussion or comment on that point. How do we really interpret some of these outlying values? Are they indicative of a possible disease or are they just the random incident that will happen occasionally?

Dr. Culver: I'm going to take the chairman's prerogative and answer that one. The first thing you do when you get a biological value that seems to be in outlying areas is repeat the test. Frequently then the problem goes away whether it was because it was a transient in the individual or whether it was a transient in the laboratory and reassurance is gained by seeing that that result was not repeated. We are getting more and more information on the variability of individuals and on the variability of responses in individuals. I think as medical surveillance continues to evolve within worksites we are going to have a lot more of that sort of information available. We are still, however, going to have to have someone give us guidance

about what those changes mean and what do the temporal relationships mean. We are seeing temporal relationships but we still don't know what the long term consequences are. Do sudden rises or falls in blood lead level have more predictive significance with regard to ultimate chronic kidney disease or do long term constant high values more likely predict chronic kidney disease? I think with this note I would like to turn this morning's session back to Dr. Thomas to conclude this session.